INDIANA UNIVERSITY

SCHOOL OF MEDICINE

July 1, 2000

Dr. Dianne Bronzert
Program Director,
National Cancer Institute
National Institutes of Health
Bethesda, MD

Dear Dr. Bronzert:

RE: Competitive renewal application RO1 CA75637

Herein, I submit an application for competitive renewal of NIH grant CA75637 entitled "Role of PTEN/PI-3 kinase/AKT signaling axis in tumor-induced angiogenesis" for your consideration. This proposal presents a completely new set of specific aims designed to determine the role and mechanism by which PTEN controls the brain tumor-induced angiogenic response. Our preliminary data provide the first direct evidence that the tumor suppressor, PTEN/AKT controls tumor-induced angiogenesis. Our proposal seeks to determine the role of the PTEN/AKT pathway in glioma-induced angiogenesis and to test the efficacy of PI-3 kinase inhibitors in the treatment of malignant glioma in our animal model.

Based on the scientific content of the proposal, we request that our grant be reviewed in Pathology B Study Section. We request that our proposal be considered by the National Cancer Institute and Neuroscience Institute for funding considerations. We appreciate your consideration of our application and look forward to seeing your evaluation of our application.

Sincerely,

Donald L. Durden, MD PhD
Department of Pediatrics, Biochemistry and
Molecular Biology
Herman B Wells Center for
Pediatrics Research
Indiana University School of Medicine
Indianapolis, IN 46204
ddurden@iupui.edu
PH: 317-278-3718
FAX: 317-286-6455

HERMAN B WELLS CENTER
FOR PEDIATRIC RESEARCH

James Whitcomb Riley
Hospital for Children
Indiana University
Medical Center
Cancer Research Institute
1044 W. Walnut Street
Room 402
Indianapolis, Indiana
46202-5225

317-274-8900
Fax 317-274-8679

AA

Form Approved Through 2/28/01
OMB No. 0925-0001

Department of Health and Human Services
Public Health Service

Grant Application

Follow instructions carefully.
Do not exceed character length restrictions indicated on sample.

LEAVE BLANK-FOR PHS USE ONLY.

Type	Activity	Number
Review Group		Formerly
Council/Board *(Month, Year)*		Date Received

1. TITLE OF PROJECT
Role of PTEN/PI-3 kinase/AKT signaling axis in tumor-induced angiogenesis

2. RESPONSE TO SPECIFIC REQUEST FOR APPLICATIONS OR PROGRAM ANNOUNCEMEN ☐ ☐ YES *(If "Yes," state number and title)*
Number: Title:

3. PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR New Investigator ☐ YES

3a. NAME *(Last, first, middle)*
Durden, Donald L.

3b. DEGREE(S)
M.D. Ph.D.

3c. SOCIAL SECURITY NO *Provide on Form Page KK*

3d. POSITION TITLE
Associate Professor

3e. MAILING ADDRESS *(Street, city, state, zip code)*
Wells Center for Pediatric Research
Indiana University School of Medicine
1044 W. Walnut, Room 468
Indianapolis, IN 46202

3f. DEPARTMENT, SERVICE, LABORATORY, OR EQUIVALENT
Pediatrics

3g. MAJOR SUBDIVISION
School of Medicine

3h. TELEPHONE AND FAX *(Area code, number and extension)*
TEL: 317-274-3718
FAX: 317-274-5378

E-MAIL ADDRESS:
ddurden@iupui.edu

4. HUMAN SUBJECTS	4a. If "Yes," Exemption no. or IRB approval date	Full IRB or Expedited Review	4b. Assurance of compliance No.	5. VERTEBRATE ANIMALS	5a. If "Yes," IACUC approval date	5b. Animal welfare assurance no.
☒ No ☐ Yes		☐		☐ No ☒ Yes	5/19/00	A4091-01

6. DATES OF PROPOSED PERIOD OF SUPPORT *(month, day, year-MM/DD/YY)*		7. COSTS REQUESTED FOR INITIAL BUDGET PERIOD		8. COSTS REQUESTED FOR PROPOSED PERIOD OF SUPPORT	
From	Through	7a. Direct Costs ($)	7b. Total Costs ($)	8a. Direct Costs ($)	8b. Total Costs ($)
04/01/01	03/31/06	291,611.	434,500.	1,548,202.	2,274,656.

9. APPLICANT ORGANIZATION
Name Indiana University
Address Research and Sponsored Programs
620 Union Drive, Room 618
Indianapolis, IN 46202

10. TYPE OF ORGANIZATION
Public: → ☐ Federal ☒ State ☐ Local
Private: → ☐ Private Nonprofit
Forprofit: → ☐ General ☐ Small Business

11. ORGANIZATIONAL COMPONENT CODE 01

12. ENTITY IDENTIFICATION NUMBER
35-6001673
DUNS NO. (if available)
00-604-6700

Congressional District
10

13. ADMINISTRATIVE OFFICIAL TO BE NOTIFIED IF AWARD IS MADE
Name William E. Farquhar
Title Asst. Vice Pres. for Research
Address Indiana University
P.O. Box 1847
Bloomington, IN 47402

Telephone (812) 855-3963
FAX (812) 855-9943

E-Mail spon2@iupui.edu

14. OFFICIAL SIGNING FOR APPLICANT ORGANIZATION
Name Mark L. Brenner, Ph.D.
Title Vice Chancellor for Res. and Grad. Ed.
Address Indiana University
Research and Sponsored Programs
620 Union Drive, Room 618
Indianapolis, IN 46202

Phone (317) 274-8285
FAX (317) 274-8744

E-Mail

15. PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR ASSURANCE:
I certify that the statements herein are true, complete and accurate to the best of my knowledge. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties. I agree to accept responsibility for the scientific conduct of the project and to provide the required progress reports if a grant is awarded as

SIGNATURE OF PI/PD NAMED IN 3a. *(In ink. "Per" signature not acceptable.)*
D Durden

DATE
6/26/00

16. APPLICANT ORGANIZATION CERTIFICATION AND ACCEPTANCE:
I certify that the statements herein are true, complete and accurate to the best of my knowledge and accept the obligation to comply with Public Health Service terms and conditions if a grant is awarded as a result of this application. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties.

SIGNATURE OF OFFICIAL NAMED IN 14. *(In ink. "Per" signature not acceptable.)*

DATE

DESCRIPTION: State the application's broad, long-term objectives and specific aims, making reference to the health relatedness of the project. Describe concisely the research design and methods for achieving these goals. Avoid summaries of past accomplishments and the use of the first person. This description is meant to serve as a succinct and accurate description of the proposed work when separated from the application. If the application is funded, this description, as is, will become public information. Therefore do not include proprietary/confidential information. DO NOT EXCEED THE SPACE PROVIDED.

Currently the treatment outcome of malignant glial brain tumors in adult and pediatric patients is poor. Mutations of the tumor suppressor, PTEN, a dual specificity protein phosphatase which dephosphorylates acidic peptides and inositol phospholipids, accompany progression of pediatric and adult brain tumors from benign to the most malignant forms. Brain tumor progression, particularly in aggressive and malignant brain tumors, is associated with augmented proliferation and the induction of angiogenesis. Our preliminary data support the hypothesis that PTEN regulates brain tumor progression by modulating the angiogenic response. U87MG glioma cells stably reconstituted with PTEN cDNA were tested for growth in a nude mouse orthotopic brain tumor model. We observed that the introduction of wild type PTEN resulted in decreased tumor growth *in vivo* and prolonged survival in mice implanted intracranially with these cells. These changes correlated with diminished phosphorylation of AKT within the PTEN-reconstituted tumor and diminished angiogenic activity, as determined by microvessel density and augmented thrombospondin 1 expression. These effects were not observed in tumors reconstituted with the G129E mutant form of PTEN in which lipid phosphatase activity is ablated. These data support our hypothesis and indicate that, in addition to the reported effects of PTEN on proliferation and cell survival, loss of PTEN regulates tumor-induced angiogenesis and the progression of gliomas to a malignant phenotype via the regulation of phosphoinositide-dependent signals. Based on our preliminary data we propose to evaluate the role of PTEN and PI-3 kinase in brain tumor progression as it relates to the angiogenic response and to determine if PI-3 kinase inhibitors can block brain tumor growth, the angiogenic response and promote survival. These experiments will provide important preclinical data to support the development of PI-3 kinase inhibitors for the treatment of malignant glial tumors associated with a deregulated PI-3 kinase/AKT signaling axis.

PERFORMANCE SITE(S) *(organization, city, state)*

Indiana University
Indianapolis, Indiana

KEY PERSONNEL. See instructions on Page 11. *Use continuation pages as needed* to provide the required information in the format shown below.

Name	Organization	Role on Project
Donald L. Durden, M.D., Ph.D.	Indiana University	P.I.
Biagio Azarrelli, Ph.D.	Indiana University	Co-PI

Type the name of the principal investigator/program director at the tope of each printed page and each continuation page. (For type specifications, see instructions on page 6.)

RESEARCH GRANT
TABLE OF CONTENTS

*Type density and type size of the entire application must conform to limites provided in instructions on page 6.

Appendix (*Five collated sets. No page numbering necessary for Appendix.*)
Number of publications and manuscripts accepted or submitted for publications (*not to exceed 10*) 10

Other items (*list*):

Color Copy of Figure 8

☒ Check if Appendix is included

DETAILED BUDGET FOR INITIAL BUDGET PERIOD
DIRECT COSTS ONLY

FROM 4/1/01 THROUGH 3/31/02

PERSONNEL (Applicant Organization Only) NAME	ROLE ON PROJECT	TYPE APPT. (months)	% EFFORT ON PROJ.	INST BASE SALARY	DOLLAR AMOUNT REQUESTED (omit cents) SALARY REQUESTED	FRINGE BENEFITS	TOTALS
Donald Durden	Principal Investigator	12	20%	$62,000	$12,400	$3,613	$16,013
Jingdong Su	Postdoc	12	100%	$35,000	$35,000	$10,199	$45,199
Chu Kang	Postdoc	12	100%	$32,000	$32,000	$9,325	$41,325
Angela Hatten	Research Analyst	12	100%	$37,900	$37,900	$11,044	$48,944
Biagio Azzarelli	Co-PI	12	10%	$113,000	$11,300	$3,293	$14,593
Iva Goss	Research Technician	12	100%	$30,000	$30,000	$7,464	$37,464
					$0	$0	$0
					$0	$0	$0
					$0	$0	$0
				SUBTOTALS →	$158,600	$44,938	$203,538

CONSULTANT COSTS		$0
EQUIPMENT (Itemize)		$0
SUPPLIES (Itemize by category)		
Animal Costs	$20,000	
Reagents and Chemicals	$3,750	
Molecular biology supplies	$7,500	
Tissue culture supplies	$13,750	
Immunohistochemical reagents and monoclonal antibodi	$12,000	
Reagents for DNA/RNA extraction	$2,000	
Embedding material for frozen sections, paraffin embedding and cryostat	$3,000	
		$62,000
TRAVEL		
One trip per year for PI to present results, and one trip per year for PI to CHLA to discuss data		$4,000
PATIENT CARE COSTS — INPATIENT		$0
PATIENT CARE COSTS — OUTPATIENT		$0
ALTERATIONS AND RENOVATIONS (Itemize by category)		$0
OTHER EXPENSES (Itemize by category)		
Publications	$2,500	
Equipment Maintenance	$2,500	
		$5,000
SUBTOTAL DIRECT COSTS FOR NEXT BUDGET PERIOD		$274,538
CONSORTIUM/CONTRACTUAL COSTS — DIRECT COSTS		$17,073
CONSORTIUM/CONTRACTUAL COSTS — INDIRECT COSTS		$0
TOTAL COSTS FOR INITIAL BUDGET PERIOD		$291,611

BUDGET FOR ENTIRE PROPOSED PROJECT PERIOD
DIRECT COSTS ONLY

BUDGET CATEGORY TOTALS		INITIAL BUDGET PERIOD *(from page 4)*	ADDITIONAL YEARS OF SUPPORT REQUESTED			
			2nd	3rd	4th	5th
PERSONNEL: *Salary & fringe benefits Applicant organization only*		$203,538	$209,644	$215,933	$222,411	$229,083
CONSULTANT COSTS		$0	$0	$0	$0	$0
EQUIPMENT		$0	$0	$0	$0	$0
SUPPLIES		$62,000	$63,860	$65,776	$67,749	$69,781
TRAVEL		$4,000	$4,120	$4,244	$4,371	$4,502
PATIENT CARE COSTS	INPATIENT	$0	$0	$0	$0	$0
	OUTPATIENT	$0	$0	$0	$0	$0
ALTERATIONS AND RENOVATIONS		$0	$0	$0	$0	$0
OTHER EXPENSES		$5,000	$5,150	$5,305	$5,464	$5,628
SUBTOTAL DIRECT COSTS		$274,538	$282,774	$291,258	$299,995	$308,994
CONSORTIUM/ CONTRACTUAL COSTS	DIRECT	$17,073	$17,585	$18,113	$18,656	$19,216
	F&A	$0	$0	$0	$0	$0
TOTAL DIRECT COSTS		$291,611	$300,359	$309,371	$318,651	$328,210

TOTAL DIRECT COSTS FOR ENTIRE PROPOSED PROJECT PERIOD (Item 8a)-> **$1,548,202**

JUSTIFICATION. Follow the budget justification instructions exactly. Use continuation pages as needed.

Budget Justification

PI: Donald L. Durden, MD PhD

Dr. Durden has overall responsibility for this investigation and will supervise all aspects of the work. He will design and perform experiments on evaluation of role of PTEN, PI-3 kinase and AKT mechanisms that control glioma -induced angiogenesis, tumor growth and animal survival. In particular, design experiments to determine the role of p53 and TSP1 in regulation of angiogenesis. He will design and implement all animal experiments to study anti-angiogenic effects of PI-3 kinase inhibitors in the orthotopic animal model. He will devote 20% of his effort to this project.

Co-investigator: Biagio Azzarelli, MD

Dr. Azzarelli is Professor of Pathology, Indiana University School of Medicine and a well establish clinical neuropathologist at this university. He is particularly interested in neuro-oncology and glial tumor pathology in pediatric and adult patients. He is the attending pathologist involved in the diagnosis and immunohistochemical analysis of brain tumors at IU School of Medicine and associated hospitals. He is director of neuropathology laboratory as part of Department of Pathology. He will be involved in the orchestration of Aim 3, the immunohistochemical analysis of PTEN, AKT and PI-3 kinase in human glioma tissues. He will orchestrate through his interaction with neurosurgery the procurement and analysis of fresh human brain tumor tissues as outlined in the last part of Aim 3. He will also serve an important role as neuropathologist in the evaluation of tumors in our orthotopic animal model (Aims 1 and 2). He will devote 10% of his effort to this project.

Research Associates:

Chu Kang, PhD Dr. Kang has been recruited to perform experiments outlined above by Dr Durden. She is a well trained in molecular biology and protein chemistry. In particular, he will perform experiments aimed at the elucidation of the important signaling events that control angiogenesis. These experiments will include biochemical analysis of AKT and PI-3 kinase pathways in our orthotopic animal model and *in vitro*. She will express mutants of AKT and PI-3 kinase in glioma cell lines and characterized effects on angiogenesis and tumor growth. She will devote 100% of his effort to this project.

Jing Dong Su, PhD Dr. Su will be responsible for performing all experiment relating the animal models used to test the effects of PTEN reconstitution on angiogenesis, tumor growth and survival analysis. He is well versed in animal experiments and molecular biology techniques. He will perform site-directed mutagenesis of PTEN and characterize glioma cells expressing these mutants. He will devote 100% effort to this project.

Technical Support:

Iva Goss Ms. Goss has 25 years experience as a neuropathology technician. She will perform the neuropathological immunohistochemical stains outlined in Aim 3. Many of these staining procedures are already established (see research plan) and yielding excellent results. Others will require initial setting up a process she has done many times. She will enter and compile data for analysis on the results of these stains in conjunction with Drs. Azarrelli and Durden. She will devote 100% effort to this project.

Angela Hatten, B.S. Ms. Hatten will perform experiments contained within Aims 1 and 2. In particular a large number of retroviral gene transduction will be required over the course of the funding period. She will transduce the glioma cell lines with mutants of PTEN, PI-3 kinase and AKT and select for stable clones. She will characterize the clones biochemically for expression of mutant protein. She will devote 100% efffort to this project.

Supply Budget

Laboratory supplies are required in the following categories and amounts:

Animal Costs	$20,000
Reagents and Chemicals	$ 3,750
Molecular biology supplies	$ 7,500
Tissue culture supplies	$13,750
Immunohistochemical reagents and monoclonal antibodies	$12,000
Reagents for DNA/RNA extraction	$ 2,000
Embedding material for frozen sections, paraffin embedding and cryostat	$ 3,000
Total	$62,000

Travel

Funding for one trip the yearly symposium on angiogenesis for the Principal Investigator is requested. Funding for one trip to Childrens Hospital Los Angeles to discuss data.

Other Expenses

$2500 is requested for maintenance of major core equipment utilized in this grant. We have found maintenance contracts to be cost-inefficient and do not carry these contracts on major equipment items unless this is the only way to assure timely repair. Thus we utilize funds on an ad hoc basis for repairs. The amount requested is an estimate based on experience. The publication funds of $2500 are requested to cover the cost of preparing and submitting work for publication.

Subcontract to University Pediatric Associates

Dr. Durden receives compensation from two sources as a faculty member of the Department of Pediatrics with the Indiana University School of Medicine. The first source is from the Indiana University Pediatrics Department, as represented in the budget under Personnel. The second source is from a third-party, non-profit corporation, University Pediatric Associates (UPA), whose mission is to support the instruction, research, and public service missions of Indiana University. This portion of salary compensation is represented in the budget under Consortium/Contractual Costs as a subcontract with UPA. It is certified that the two sources of income comprise a single compensation package for this faculty.

PROPOSAL TO
INDIANA UNIVERSITY

Title: Role of PTEN/PI-3 kinase/AKT signaling axis in tumor-induced angiogenesis

Principal Investigator: Donald L. Durden, M.D., Ph.D.

Mailing Address:
Indiana University Medical Center
Wells Center for Pediatric Research
702 Barnhill Drive, Room 2600
Indianapolis, IN 46202-5225

Project Duration: 4/1/01-3/31/06

Amount Requested:
$17,073 Year 1
$90,643 All Years

Applicant Institution:
University Pediatric Associates, Inc.
702 Barnhill Drive, Room 5900
Indianapolis, IN 46202-5225
(317) 274-7810

Financial Officer:
James A. Lemons, M.D.
Secretary-Treasurer
University Pediatric Associates, Inc.
Indianapolis, IN 46202-5225

Identification Number: 23 7328642

Individual Authorized to Sign for the Institution:

_____
Richard L. Schreiner, M.D.
Chairman of the Board

SUBCONTRACT TO UNIVERSITY PEDIATRIC ASSOCIATES

STATEMENT OF INTENT TO ESTABLISH A CONSORTIUM AGREEMENT

Date: June 27, 2000

Grant Number: 2R01 CA75637

Application Title: Role of PTEN/PI-3 kinase/AKT signaling axis in tumor-induced angiogenesis

Proposed Project Period: 4/1/01-3/31/06

"The appropriate programmatic and administrative personnel of each institution involved in this grant application are aware of the NIH consortium grants policy and will establish the necessary inter-institutional agreement(s) consistent with that policy.

"Further, (1) the prospective lower tier participant certifies, by submission of this proposal, that neither it nor its principals are presently debarred, suspended, proposed for debarment, declared ineligible, or voluntarily excluded from participation in this transaction by any Federal department or agency, and (2) where the prospective lower tier participant is unable to certify to any of the statements in this certification, such prospective participant shall attach an explanation to this proposal."

Indiana University
Applicant Institution

Principal Investigator

Official Authorized to Sign for Institution
Mark L. Brenner, Ph.D.
Vice Chancellor for Research and and Graduate Education

University Pediatric Associates
Consortium Institution

Principal Investigator

Official Authorized to Sign for Institution
Richard L. Schreiner, M.D.
Chairman of the Board

SUBCONTRACT TO UNIVERSITY PEDIATRIC ASSOCIATES Principal Investigator/Program Director *(Last, first, middle)*: Durden, Donald L.

DETAILED BUDGET FOR INITIAL BUDGET PERIOD
DIRECT COSTS ONLY

FROM 4/1/01 THROUGH 3/31/02

PERSONNEL (Applicant Organization Only) NAME	ROLE ON PROJECT	TYPE APPT. *(months)*	% EFFORT ON PROJ.	INST BASE SALARY	DOLLAR AMOUNT REQUESTED (omit cents) SALARY REQUESTED	FRINGE BENEFITS	TOTALS
Donald Durden	Principal Investigator	12	20%	$79,300	$15,860	$1,213	$17,073
					$0	$0	$0
					$0	$0	$0
					$0	$0	$0
					$0	$0	$0
					$0	$0	$0
					$0	$0	$0
					$0	$0	$0
				SUBTOTALS →	$15,860	$1,213	$17,073

CONSULTANT COSTS		$0
EQUIPMENT (Itemize)		$0
SUPPLIES (Itemize by category)		$0
TRAVEL		$0
PATIENT CARE COSTS	INPATIENT	$0
	OUTPATIENT	$0
ALTERATIONS AND RENOVATIONS (Itemize by category)		$0
OTHER EXPENSES (Itemize by category)		$0
SUBTOTAL DIRECT COSTS FOR NEXT BUDGET PERIOD		**$17,073**
CONSORTIUM/CONTRACTUAL COSTS	DIRECT COSTS	$0
	INDIRECT COSTS	$0
TOTAL COSTS FOR INITIAL BUDGET PERIOD	*(Item 7a, Face Page)* →	**$17,073**

BUDGET FOR ENTIRE PROPOSED PROJECT PERIOD
DIRECT COSTS ONLY

BUDGET CATEGORY TOTALS		INITIAL BUDGET PERIOD *(from page 4)*	ADDITIONAL YEARS OF SUPPORT REQUESTED			
			2nd	3rd	4th	5th
PERSONNEL: *Salary & fringe benefits Applicant organization only*		$17,073	$17,585	$18,113	$18,656	$19,216
CONSULTANT COSTS		$0	$0	$0	$0	$0
EQUIPMENT		$0	$0	$0	$0	$0
SUPPLIES		$0	$0	$0	$0	$0
TRAVEL		$0	$0	$0	$0	$0
PATIENT CARE COSTS	INPATIENT	$0	$0	$0	$0	$0
	OUTPATIENT	$0	$0	$0	$0	$0
ALTERATIONS AND RENOVATIONS		$0	$0	$0	$0	$0
OTHER EXPENSES		$0	$0	$0	$0	$0
SUBTOTAL DIRECT COSTS		$17,073	$17,585	$18,113	$18,656	$19,216
CONSORTIUM/ CONTRACTUAL COSTS	DIRECT	$0	$0	$0	$0	$0
	F&A	$0	$0	$0	$0	$0
TOTAL DIRECT COSTS		$17,073	$17,585	$18,113	$18,656	$19,216
TOTAL DIRECT COSTS FOR ENTIRE PROPOSED PROJECT PERIOD (Item 8a)->					**$90,643**	

JUSTIFICATION. Follow the budget justification instructions exactly. Use continuation pages as needed.

The faculty of the Department of Pediatrics at the Indiana University School of Medicine receive compensation from both Indiana University (I.U.) and University Pediatric Associates, Inc. (UPA). The salary from I.U. contains retirement, health, life and disability benefit options. The salary from University Pediatric Associates, Inc. includes only Social Security and Medicare tax in the fringe benefit cost. The salary and fringe benefit component of the UPA salary is included with the attached subcontract proposal. The combined salary represents both proportional effort and compensation for this research project as outlined in the primary proposal submitted through Indiana University. The combining of these salaries represents a savings in the total cost due to the substantial reduction in costs for University fringe benefits. This change in compensation has been reviewed by the Department of Health and Human Services with the acceptance provided from the Office of Secretary to include compensation provided by the faculty practice plan as a subcontract in processing proposals for research grants. Notification has also been provided to the Indiana State Board of Accounts at the Central States Branch Office in Indianapolis.

BIOGRAPHICAL SKETCH

Give the following information for the key personnel and consultants and collaborators. Begin with the principal investigator/program director. Photocopy this page for each person.

NAME	POSITION TITLE
Donald L. Durden, M.D., Ph.D.	Associate Professor of Pediatrics & Biochemistry

EDUCATION *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	FIELD OF STUDY
University of South Florida, Tampa, FL	B.S.	1977	Microbiology/Zoology
University of Miami School of Medicine, Miami, FL	Ph.D.	1983	Microbiology/Immunology
University of Miami School of Medicine, Miami, FL	M.D.	1985	Medical Doctor
Childrens Hospital of Medical Center, Seattle, WA	Fellow	1987-1988	Pediatric Hem/Onc
Fred Hutchinson Cancer Research Center, Seattle, WA	Fellow	1988-1992	Molecular/Cell Biology

RESEARCH AND/OR PROFESSIONAL EXPERIENCE: Concluding with present position, list in chronological order previous employment, experience, and honors. Key personnel include the principal investigator and any other individuals who participate in the scientific development or execution of the project. Key personnel typically will include all individuals with doctoral or other professional degrees, but in some projects will include individuals at the masters or baccalaureate level provided they contribute in a substantive way to the scientific development or execution of the project. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. DO NOT EXCEED TWO PAGES.

Professional Experience:

1999-Present — Associate Professor, Pediatrics and Biochemistry, Herman B. Wells Center for Pediatric Research, Indiana University School of Medicine, Indianapolis, Indiana.

1993-Apr. 1999 — Assistant Professor, Division of Hematology-Oncology, Department of Pediatrics, Childrens Hospital Los Angeles/University of Southern California School of Medicine, Los Angeles, California.

1989-1992 — Postdoctoral fellowship, Fred Hutchinson Cancer Research Center, Seattle, WA, Role of tyrosine phosphorylation in myeloid signal transduction, Jonathan Cooper, Supervisor.

1979-1985 — Graduate/Medical Student Research, Department of Microbiology and Immunology, University of Miami School of Medicine, Miami, FL. Isolation and characterization of Vibrio L-asparaginase. J.A. Distasio, Advisor.

SELECTED PUBLICATIONS:

1. Charyulu, V., Sigel, M.M., **Durden, D.L.**, and Lopez, D.M. Mouse mammary tumor virus (MMTV) antigen(s) are present on B-lymphocytes of Balb/c mice. *Int J Cance,r* 24:813-818, 1979.

2. **Durden, D.L.** and Distasio, J.A. Comparison of the immunosuppressive effects of L-asparaginases from *Escherichia coli* and *Vibrio succinogenes*. *Cancer Res,* **40**:1125-1129, 1980.

3. **Durden, D.L.** and Distasio, J.A. Characterization of the effects of *Escherichia coli* asparaginase and a glutaminase-free asparaginase from *Vibrio succinogenes* on specific cell-mediated cytotoxicity. *Int J Cancer,* 27:59-65, 1981.

4. Distasio, J.A., **Durden, D.L.**, Paul, R.D., and Nadji, M. Alteration in spleen lymphoid populations associated with specific amino acid depletion during L-asparaginase treatment. *Cancer Res,* **42**:252-258, 1982.

5. Distasio, J.A., Salazar, A.M., Nadji, M., and **Durden, D.L.** Glutaminase-free asparaginase from *Vibrio succinogenes*: an antilymphoma enzyme lacking hepatoxicity. *Int J Cancer,* 30:343-347, 1982.

6. **Durden, D.L.**, Salazar, A.M., and Distasio, J.A. Kinetic analysis of hepatotoxicity associated with antineoplastic asparaginases. *Cancer Res.,* 43:1602-1605, 1983.

7. Kazlauskas, A., **Durden, D.L.**, and Cooper, J.A. Functions of the major tyrosine phosphorylation site of the PDGF receptor beta subunit. *Cell Regul,* 2:413-425, 1991.

8. **Durden, D.L.**, Rosen, H., and Cooper, J.A. Serine/threonine phosphorylation of the γ subunit after activation of the high-affinity Fc receptor for immunoglobulin G. *Biochem, J* **299**:569-577, 1994.

9. **Durden, D.L.**, Rosen, H., Michel, B.R., and Cooper, J.A. Protein tyrosine phosphatase inhibitors block myeloid signal transduction through the FcγRI receptor. *Exp Cell Res,* **211**:150-162, 1994.

10. **Durden, D.L.**, and Liu, Y.B. Protein-tyrosine kinase $p72^{syk}$ in FcγRI receptor signaling. *Blood,* **84**:2102-2108 (rapid communication), 1994.

11. **Durden, D.L.**, Kim, H.M., Calore, B., and Liu, Y.B. The FcγRI receptor signals through the activation of *hck* and MAP kinase. *J Immun,* **154**:4039-4047, 1995.

12. Arditi, M., Zhou, J., Martine, T., **Durden, D.L.**, Stins, M., and Kim, K-S. Lipopolysaccharide stimulates the tyrosine phosphorylation of mitogen-activated protein kinases, p44, p42, and p38 in vascular endothelial cells in a soluble CD14-dependent manner: Role of protein tyrosine phosphorylation in lipopolysaccharide-induced stimulation of endothelial cells. *J Immun,* **155**(8):3994-4003, 1995.

13. Park, R.K., Liu, Y.B., and **Durden, D.L.** A role for Shc, Grb2 and Raf-1 in FcγRI signal relay. *J Biol Chem,* **271**:13342-13348, 1996.

14. Taylor, N., Jahn, T., Smith, S., Uribe, L., Liu, Y.B., **Durden, D.L.**, and Weinberg, K. Differential activation of the tyrosine kinases ZAP-70 and SYK in FcγRI signaling. *Blood,* **89**:388-396, 1997.

15. Epstein, A., Liu, M., Liu, Y.B. and **Durden, D.L.**, Protein tyrosine phosphtase inhibitors in FcγRI induced myeloid oxidant signal transducton. *Exp. Cell Res,* **237**:288-295, 1997.

16. Park, R.K., Liu, Y.B., Kyono, W., and **Durden, D.L.** CBL-GRB2 adapter protein interaction in immunoreceptor tyrosine activation motif (ITAM) signaling. *J. Immunol,* **160**:5018-5027, 1998.

17. Chu, J., Liu, Y, Koretzky, G.A. and **Durden, D.L.** SLP-76-CBL-Grb2-Shc interactions in FcγRI signaling. *Blood,* **92**:1697-1706, 1998.

18. Kyono, W. T., De Jong, R., Park, R. K., Liu, Y.B., Heisterkamp, N., Groffen, J. and **Durden, D.L.** Differential interaction of Crkl with Cbl or C3G, Hef-1 and γ-subunit ITAM in myeloid FcγRI signaling. *J. Immunol,* **161**:5555-5563,1998.

19. Izadi, K., Erdreich-Epstein, A., Liu, Y., and **Durden, D.L.** Characterization of Cbl-Nck and Nck-Pak1 interactions in myeloid FcγRII signaling. *Exp Cell Res,* **245**:330-342, 1998.

20. Erdreich-Epstein, A., Liu, M. Kant, A., Izadi, K., Nolta, J. and **Durden, D.L.** CBL functions downstream of Src kinases in FcγRI signaling in primary human macrophages *J. Leuk. Biol,* **65**:523-534, 1999.

22. Park, Rae-Kil, Izadi, K., Deo, Y.M., Liu, Y.B. and **Durden, D.L.** Role of Src in the modulation of multiple adaptor proteins in FcαRI oxidant signaling. *Blood,* **94**:2112-2120, 1999.

23. Park, R.K., Erdreich-Epstein, A., Liu, M., Izadi, K.D., and **Durden, D.L.** High affinity IgG receptor activation of Src family kinases is required for modulation of the Shc-Grb2-Sos complex and the downstream activation of the nicotinamide adenine dinucleotide phosphate (reduced) oxidase. *J. Immunol,* **163**:6023-6034, 1999.

24. Erdreich-Epstein, A., Shimada, H., Groshen, S., Liu, M., Metelitsa, L., Kim, K.S., Stins, M., Seeger, R.C. and **Durden, D.L.** Integrins αvβ3 and αvβ5 are expressed by endothelium of high-risk neuroblastoma and their inhibition is associated with increased endogenous ceramide. *Cancer Research,* **60**:712-721, 2000.

25. Su, J.D., Wigler, M.H., Tonks, N.K., **Durden, D.L.** PTEN controls the growth and angiogenic response of malignant glimoas. *Nat. Genet.* Submitted. 2000.

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed on Form Page 2.
Photocopy this page or follow this format for each person.

NAME	POSITION TITLE
Biagio Azzarelli	Professor

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training).*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
University of Chile, Santiago, Chile	Baccalaureate	1960	Biology
University of Chile School of Medicine, Santiago, Chile	M.D.	1967	Biology
Case Western Reserve University, Cleveland, OH	Resident	1975	Pathol/Neuropathol

RESEARCH AND PROFESSIONAL EXPERIENCE: Concluding with present position, list, in chronological order, previous employment, experience, and honors. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. If the list of publications in the last three years exceeds two pages, select the most pertinent publications. **DO NOT EXCEED TWO PAGES.**

Fellow, Pathology, Case Western Reserve University School of Medicine, Cleveland, OH, 1973-1976
Instructor, Department of Neurpathology, Case Western Reserve University School of Medicine, Cleveland, OH, 1976-1977
Assistant Professor, Department of Pathology, University of Maryland School of Medicine, Baltimore, MD, 1977-1978
Director, Neuropathology and Legal Medicine, Maryland State Department of Health and Mental Hygiene, Baltimore, MD, 1977-1979
Visiting Scholar, Department of Biology, Indiana University, Bloomington, IN, 1989-1990
Associate Professor of Pathology (Neuropathology), Indiana University School of Medicine, Indianapolis, IN, 1979-1995
Professor of Pathology (Neuropathology), Indiana University School of Medicine, Indianapolis, IN, 1995-date
Professor of Neurology, Indiana University School of Medicine, Indianapolis, IN, 1995-date
Professor of Neurosurgery, Indiana University School of Medicine, Indianapolis, IN, 1997-date

PUBLICATIONS: (Selected from 95 papers and review articles)

1. Collins M, Azzarelli B, West K, Chong S, Maguiness K, Stevens J (1996) Neuropathy and vasculopathy in colonic strictures from children with cystic fibrosis. J Ped Surg 31:945-950
2. Hari J, Azzarelli B, Caldemeyer K (1996) Malignant fibrous histicytoma. Ped Neurosurg 24:160-166
3. Yee RD, PurvinVA, Azzarelli B, Nelson PB (1996) Intermittent diplopia and stravismus caused by ocular neuromyotonia. Tr AmSoc XCIV 207-226
4. Wolfla CE, Azzarelli B, Shah MV (1997) Solitary intradural extramedullary ependymoma of the thoracic spin: Case Report. J Neurosurg 87:643
5. Kotylo PK, Robertson P, Finemberg NS, Azzarelli B (1997) Flow cytometric DNA analysis of pediatric cerebral ependymomas. Arch Pathol 121:1255-1258
6. Goebel HH, D'Agostino AN, Wilson J, Cole G, Foroud T, Koller D, Farlow M, Azzarelli B, Muller J (1997) Spheroid body myopathy revisited. Muscle & Nerve 20:1127-1136
7. Sokol DK, Azzarelli B, Smith RR, Kassing L, Pascuzzi R (1998) Primary intravascular lymphomatosis associated with mycobacterium marinum. J Neuroimag 8:47-49
8. Salanova V, Markand O, Worth R, Smith R, Wellman H, Hutchins G, Park HM, Ghetti B, Azzarelli B (1998) FDG-PET and volumetric MRI in temporal lobe epilepsy (TLE) relationship to febrile seizures and hippocampal sclerosis. Acta Neurol Scan 98:146-153
9. Salanova V, Markand O, Worth R, Garg B, Patel H, Asconope J, Park H-M, Hutchins GD, Smith R, Azzarelli B (1999) Presurgical evaluation and surgical outcome of temporal lobe epilepsy. Pediatr Neurol 20:179-184
10. Martidis A, Yee RD, Azzarelli B, Biller J (1999) Neuro-ophthalmic, radiographic and pathologic manifestations of adult-onset Alexander disease. Arch Ophthalmol 117:265-267
11. Qu C-K, Yu W-M, Azzarelli B, Feng G-S (1999) Genetic evidence that Shp-2 tyrosine phosophatase is a signal enhancer of the epidermal growth factor receptor in mammals. Proc Natl Acad Sci USA 96:8528-8533
12. Qu C-K, Yu W-M, Azzarelli B, Cooper S, Broxmeyer HE, Feng G-H (1999) Biased suppression of hematopoiesis and multiple developmental defects in Chimeric mice containing Shp-2 mutant cells. Mol Cell Biol 18:6075-6082

This page intentionally left blank.

OTHER SUPPORT

DURDEN, DONALD L

Current

RPG-98-244-01-LBC (D. Durden) American Cancer Society "Cbi-adapter protein interaction in regulation of RAS"	7/1/98-6/30/01	20% $110,000
(R. Seeger) NIH "Biology and Therapy of High-Risk Neuroblastoma" D. Durden, PI of Project 2	4/1/00-3/31/05	10% $76,202

Pending

None

Overlap

None

AZARELLI, BIAGIO

Current

None

Pending

None

Overlap

None

RESOURCES

FACILITIES: Specify the facilities to be used for the conduct of the proposed research. Indicate the performace sites and describe their capacities, pertinent capabilities, relative proximity, and extent of availability to the project. Under "Other", identify support services such as machine shop, electronics shop, and specify the extent to which they will be available to the project. Use continuation pages if necessary.

Laboratory: Dr. Durden's 900 square foot laboratory is located in the Cancer Research Institute (CRI). The state-of-the-art research building opened in June 1997 and houses the Herman B Wells Center for Pediatric Research, the Walther Oncology Center, and the Indiana University Cancer Center. Additional Wells Center space is located in a group of contiguous laboratories in Riley Hospital, which is connected by tunnels and an above ground pedestrian bridge to the CRI. In total, the Herman B Wells Center for Pediatric Research has 22 laboratories and supporting office and administrative space located in 27,500 square feet. In addition, the Center houses 4 common equipment rooms which provide space for large and shared equipment, 2 common tissue culture rooms, three dark rooms, 2 cold rooms and one service equipped with dishwasher/dryer and autoclave for glassware and media preparation.

Clinical:

Animal: The animal facilities available to Dr. Durden are maintained by the Laboratory Animal Resource Center at Indiana University, and includes 8 dedicated rooms for housing mice and 137 CS-irradiator located in contiguous space. 1/8 of the rooms is dedicated to murine bone marrow transplant studies. 1/8 of the rooms is dedicated to transgenic mice, and 1/8 of the rooms is dedicated to ES-derived chimeric mice. A new transgenic facility opened in the Cancer Research Institute. This facility includes five additional rooms, a procedure room, and two microinjection facilities, and laminar flow caging used for NOD/SCID colony.

Computer: Dr. Durden's office and laboratory are equipped with Macintosh and IBM computers . All computers are connected to the Wells Center computer network (Novell-based), and through this network to other University computers and the Internet. MacVector sequence analysis program is available.

Office: Dr. Durden has a 160 sq. ft. office immediately adjacent to her laboratories.. Desk space is available for research fellows and technical staff in the laboratory. Secretarial support is available as needed.

Other ( ): The CRI houses conference rooms on each floor. The CRI also contains a state-of-the-art auditorium, which seats 120 people. The Wells Center houses two conference rooms, each with adequate seating for ~35 individuals. These rooms are equipped with slide and overhead projectors. These rooms house the journal collection, which includes issues to one year old of 25 journals. The Medical School library is located directly across the street for older issues. The administrative support space is located within the Wells Center.

MAJOR EQUIPMENT: List the most important equipment items already available for this project, noting the location and pertinent capabilities of each.

The common equipment areas are conveniently located within several feet of all laboratories and contain all necessary equipment for molecular and cell biology research including ultracentrifuges, scintillation counter, gamma counter, gel dryers, sonicator, bacterial incubators, FACScan flow analysis machine, -80° freezers, and multiple low and high speed centrifuges. Much of the core equipment has been provided by the Howard Hughes Medical Institute and by start-up funds from the Riley Memorial Association. Tissue culture core areas contain biological hoods, incubators, tissue culture microscopes, refrigerators and centrifuges. Dark rooms contain cameras and UV light boxes for gel photography and a Kodak x-omat for x-ray film processing. One dark room is set up for the fluorescence microscopy and contains a Nikon axiophot scope and camera. One dark room is set up for *in situ* hybridization studies. Cold rooms contain centrifuges, FPLC apparatus, and media storage areas.

Specific Aims:

Our basic science focus has changed significantly for our competitive renewal grant application. This is based on an important new observation made in our laboratory over the past 2 years. Mutations in PTEN, a dual specificity protein and lipid phosphatase, have been implicated in the malignant progression of adult and pediatric glial tumors (1, 2). In an orthotopic brain tumor model, we observe that PTEN/PI-3 kinase pathway exerts control over angiogenesis, malignant glioma growth and animal survival. Herein we propose to determine the role of PTEN and PI-3 kinase in the control of tumor-induced angiogenesis as defined in the new specific aims:

Aim 1. We will use our orthotopic nude mouse brain tumor model to determine the role of PTEN and specific mutations in PTEN in the angiogenic response of malignant gliomas. We will generate mutations in PTEN, stable clones of glioma tumor cells expressing these mutants and test these cells in our orthotopic nude mouse model for effects on angiogenesis, tumor growth and animal survival. We will explore the mechanism by which PTEN regulates thrombospondin 1 expression in the control of angiogenesis. We will evaluate the effect of PTEN on MDM2/p53 signaling axis.

Aim 2. We will investigate the role of PI-3 kinase and/or AKT kinase in control of tumor-induced angiogenesis, thrombospondin expression, tumor growth and animal survival.

Aim 3. We will determine if PI-3 kinase inhibitors have effects on glioma growth *in vitro* or *in vivo* and whether and how these inhibitors affect the process of angiogenesis and mortality *in-vivo*. Test PI-3 kinase inhibitors (LY294002) for potential therapeutic activity in our orthotopic brain tumor model. We will evaluate adult and pediatric brain tumor tissue for evidence of altered PI-3 kinase/AKT signaling and for mutations in PTEN by RT-PCR, SSCP and immunohistochemical analysis.

Background and Significance.

Brain tumors are the most common solid tumor in children and account for 30 percent of cancer related deaths in childhood. Moreover, 40,000 adults are diagnosed with a malignant brain tumor within the U.S. per year and the mortality from these tumors is >90%. Hence the therapy for malignant human brain tumors is in need of significant improvement. Understanding the molecular basis for brain tumor progression is critical for the development of effective therapies for this disease. Recent experiments have implicated a number of oncogenes and suppressor oncoproteins in the development of brain tumors (EGF receptor, p53, MDM2 and PTEN). PTEN is a dual specificity phosphatase which dephosphorylates acidic peptides and inositol phospholipids and has been identified as a tumor suppressor gene in many tumor types. The PTEN gene is mutated in 26% of high-grade pediatric gliomas and 50% or adult malignant gliomas (1, 2). Currently there is a significant interest in determining the mechanisms by which mutations in PTEN may result in the initiation and progression of malignant glial tumors. Part of this intense interest relates to the current development of PI-3 kinase inhibitors which in some ways are expected to mimic the effects of PTEN reconstitution.

Brain tumor biology, signaling and angiogenesis.

Most of the information, which has been assembled on the mechanism(s) of glial transformation, comes from the study of glioma cell lines. Currently there are more than 25 human glioblastoma-derived cell lines which have been extensively studied (3, 4). It is clear that mutations in multiple oncogenes and suppressor oncogenes cooperate to drive the malignant progression of human brain tumors. Gene duplications involving MDM2 (murine double minute), a p53 associated protein and the EGF receptor contribute to malignant phenotype. Mutations within tumor suppressor genes contribute to malignant transformation of glial tumors including p53 and PTEN. Each glioma-derived cell line has a different complement of oncogenic mutations contributing to different phenotypes (angiogenesis, migration, invasion, proliferation, apoptosis, etc.). We will utilize specific human glioma cell lines (U87MG, U373MG and U251MG) in our experiments based on knowledge of oncogenes inherent to them. All of

PTEN is mutated during progression of gliomas.

Mutations in PTEN accompany progression of brain tumors from grade I/II to malignant grade III and IV *in vivo* (1, 2). Tumor progression is associated with angiogenesis, the formation of new blood vessels from existing vascular structures, with increases in microvessel density and increased invasion of tumor cells into brain parenchyma (28-30). During angiogenesis endothelial cells are induced to degrade the basement membrane of existing vessels, break away and migrate to the site of the tumor, where they proliferate to form linear structures which differentiate to form blood vessels. Factors that control angiogenesis include growth factors, matrix metalloproteinases, plasminogen activators, thrombospondins, integrins $\alpha v\beta 3$, $\alpha v\beta 5$ and $\alpha 5\beta 1$ (31). Malignant tumors, which tend to induce a more robust angiogenic response as compared to their benign counterparts, are classified histopathologically by the presence or absence of high microvessel counts (microvessel density). It has been suggested that tumor progression may involve an "angiogenic switch" necessary for tumor growth and metastasis. Currently the intracytoplasmic signaling event(s), which control the "angiogenic switch", are not known. Regulation of PI-3 kinase-dependent signals, including activation of AKT by Vascular Endothelial cell Growth Factor (VEGF) and its receptor, the protein tyrosine kinase Flt-1, has been implicated in brain tumor angiogenesis (32). It has been shown in the chicken chorioallantoic membrane (CAM) model that PI-3 kinase-dependent pathways may regulate angiogenesis and VEGF expression in endothelial cells (33). In addition, overexpression of PTEN inhibited angiogenesis within the yolk sac of the chicken embryo. Furthermore, it has been observed that prostate tumor specimens that contain PTEN mutations have higher microvessel counts than tumors expressing wild type PTEN (34). However, whether PTEN is causally linked to induction of angiogenesis by the tumor cell remains unproven. These and other observations led us to hypothesize that PTEN may control the angiogenic response and contribute to the high mortality associated with malignant brain tumors.

Preliminary Data/Progress Report:

Summary of Previous Specific Aims.

Progress Report. (Phase II Clinical Trial of polyICLC)

Our previous grant was designed to determine if the biologic response modifier, polyICLC would have activity in treatment of pediatric brain tumors. We are close to completing this ongoing Phase II clinical trial of polyICLC for treatment of recurrent and high-grade brain tumors in children (continued accrual of patients, particularly in the glioma disease category). Importantly, we have observed clinical responses to this agent in high-grade and low-grade glioma patients, enough to justify the continued study of this agent. If the response continue as observed, this agent will be considered for a Phase III trial by the Children's Oncology Group, Brain Tumor Strategy Group, for high and low grade gliomas after standard therapy as a biologic arm to therapy i.e. maintenance phase. As far as mechanisms for polyICLC action in brain tumor growth and angiogenesis, we continue to examine the molecular basis for polyICLC action as an antitumor agent in our orthotopic brain tumor model. For our competitive renewal, we present data which supports an entirely new set of specific aims.

Phase II clinical trial of polyICLC in high and low grade glioma patients.

Based on responses observed in pediatric and adult patients with high grade and low grade gliomas we plan to continue our clinical trial with the targeted accrual of glioma patients. No major changes in the clinical protocol design are anticipated and the clinical trial is proceeding well with minimal to no toxicity seen. The study continues to accrue patients in cohort 1 and 2.

Patient Entry/Response Data.

A total of 45 patients have been entered on study to date (7 within the last year) of which 32 are evaluable. Of the evaluable patients; eight had newly diagnosed gliomas and the remaining 24 had

recurrent brain tumors. Four patients continue on study to date (1pt within 2 months; 1 pt within 5 months and 2 pts greater than 20 months). Two patients continue to receive the drug (greater than 2 years of treatment) off study and 1 additional patient completed 2 years of therapy and went off study.

In cohort 1 (Recurrent high grade gliomas), a total of 16 patients have been entered. 11 are evaluable, 1 is too early to evaluate and 4 are inevaluable. Of the evaluable patients, 1 patient had a documented PR to therapy and another patient has had stable disease for greater than 20 months. Another 2 patients treated at National Childrens Medical Center have shown response for total of 4/11 for response rate of 36%. There has been sufficient response in this cohort of patients to continue accrual. The target accrual would be an additional 13-26 patients depending on response rate.

In cohort 2, low grade gliomas, we have enrolled 4 patients with 2 long term responses (50% response rate). In this cohort we define long term response as PR or stable disease for length of treatment (2 years). We need additional accrual (13-26 total patients) in this cohort to answer question.

In cohort 3 (Newly diagnosed brain stem gliomas), a total of 13 patients have been entered. 8 are evaluable and 5 are inevaluable. Of the evaluable patients, all patients have progressed within 2 to 7 months of going on study. After discussion with the statistician, we plan to close this cohort to patient entry due to a lack of response to the therapy. Hence we conclude that polyICLC has no activity in the treatment of childhood brain stem gliomas.

Cohorts 4 and 5 include other recurrent brain tumors and neurofibromatosis-related brain tumors respectively. 9 patients are evaluable at this time with no responses noted. These will not be considered as a statistical endpoint for the study.

Toxicity.

There has been no dose limiting toxicity experienced over the last 3 years. The study was amended once within the last year to increase the treatment dose of the drug to 30ug/kg/dose and to add patients diagnosed with neurofibromatosis type 1 and 2.

Preliminary Data to Support Our New Specific Aims:

PTEN/PI-3 kinase controls brain tumor angiogenesis and progression.

We have performed experiments in our orthotopic model which indicate that PTEN controls angiogenesis, tumor growth and survival of mice implanted orthotopically with U87MG tumor cells (Figure 2-5, below). Many questions remain regarding the mechanism by which PTEN exerts its control over the angiogenic process.

Orthotopic Brain tumor model.

To determine whether PTEN exerts control over angiogenesis and/or growth of glial tumors, we developed an orthotopic brain tumor model in which PTEN-deficient cells were genetically manipulated *in vitro* and then stereotactically injected into the frontal cerebral cortex of nude mice. The U87MG cell line is derived from a patient diagnosed with glioblastoma multiforme, a highly malignant and uniformly fatal brain tumor. This tumor and other human glioblastomas and glioblastoma cell lines have been shown to contain a mutation in both PTEN alleles (U87MG cells have a homozygous mutation in PTEN resulting in a null genotype) (13). In light of these observations, we reconstituted the PTEN gene in the parental U87MG (U87) cells. In this model 100% of mice implanted intracranially with the parental U87 cells display a highly invasive and angiogenic pattern of brain tumor growth that results in mortality within 25-27 days. Stable derivatives of the parental U87 cells were generated following transduction with retroviruses encoding cDNA for wild type PTEN or specific mutants of the phosphatase. In particular, we used missense mutations in the PTP signature motif to ascertain the

importance of the enzymatic activity of PTEN to its tumor suppressor function. This included R130M, in which all phosphatase activity is abrogated, and G129E, which has been confirmed in Cowden disease and endometrial cancer and in which the activity toward inositol phospholipids is severely attenuated but protein phosphatase activity is essentially intact (12, 35). Tumor cells were characterized biochemically for levels of activated AKT, growth *in vitro* and PTEN expression (Figure 2). Anti-PTEN blots confirmed that the parental U87 cells do not express PTEN and that following reconstitution of PTEN expression, U87 cells express significant and comparable amounts of the wild type or mutant phosphatase protein. Expression of wild type PTEN, to levels similar to those observed in a mouse brain lysate, suppressed the activated state of AKT observed in PTEN-deficient U87 cells (Figure 2A, lanes 3 & 5). Following expression of the R130M and G129E mutant forms of PTEN, the levels of phospho-AKT were similar to those observed in the parental U87 cells (Figure 2A, lanes 1, 2 & 4), suggesting that the lipid phosphatase activity of
PTEN was essential for the effects on the PIP_3-dependent activation of AKT. Importantly, the growth of the different PTEN-expressing U87 cell lines *in vitro* was similar in 2, 5 and 10% fetal bovine serum (data not shown and Figure 2B). Therefore, these data support the validity of further comparison of these cell lines in our *in vivo* model (data shown below).

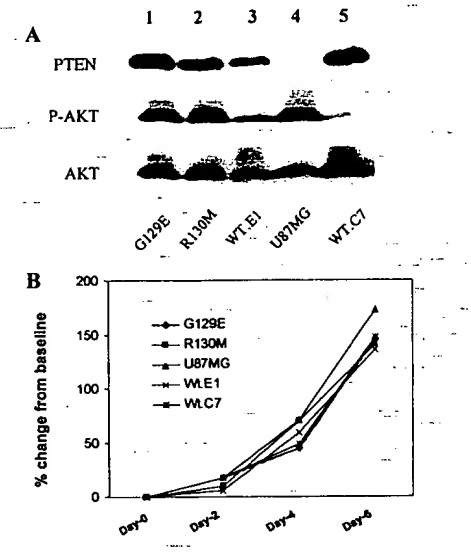

Figure 2. Stable expression of PTEN and PTEN mutants in U87MG cells regulates AKT. (A) Cell lysates from the U87MG (U87) cell line and U87 cells infected with a retroviral vector encoding PTEN (pBabe-Puro-PTEN) or mutants of PTEN (pBabe-Puro-PTEN-G129E or R130M) were resolved by SDS-PAGE, equal amounts of proteins were loaded per lane and immunoblotted with antisera to PTEN, phospho-AKT and total AKT, and visualized by enhanced chemiluminescence. The basal levels of PTEN (top), phosphorylated AKT (ser 473) (middle) and total AKT (bottom) are shown. The status of the PTEN gene in each stable cell line was designated as: WT.E1 and WT.C7 two separate clones expressing wild type PTEN, R130M and G129E are mutated PTEN, R130M is inert as both a protein and a lipid phosphatase. The G129E PTEN can dephosphorylate acidic phosphopeptides, but cannot dephosphorylate lipid substrate, PIP3. The U87MG (U87) cell line is the parental cell line isolated from a human glioblastoma multiforme patient. **(B)** Comparison of *in vitro* growth of U87MG cells transduced with mutants of PTEN. Equal number of cells ($1x10^5$) were incubated in RPMI + 10% FBS for different times and cell numbers were quantitated by direct cell counting.

Effect of PTEN on tumor growth.

To determine the effect of PTEN reconstitution on tumor growth *in vivo*, we implanted athymic nude mice with PTEN reconstituted U87 glioma cells both subcutaneously and by intracranial injection. Production of subcutaneous tumors allowed us to monitor the size of the tumor and to perform biochemical analysis of tumor tissue for PTEN expression and levels of AKT activation without significant contamination from other tissues. Tumor tissue blocks, processed for hematoxylin and eosin staining, confirmed that >95% of tissue comprised tumor cells free of dermal or subdermal tissue. We compared the levels of PTEN in tumor tissue and numerous normal tissues within the athymic nude mouse. Using anti-PTEN antisera, we detected the expression of PTEN in all tissues, with the exception of skeletal and heart muscle (data not shown), but no PTEN was detected in the parental U87-derived tumor tissue (Figure 3C, lane 4). These results indicate that the tumor tissue sampled represents predominantly tumor cell-derived proteins. As observed in the cell lines grown *in vitro*, subcutaneous tumors derived from U87 cells reconstituted with mutant or wild type PTEN displayed similar levels of PTEN expression (Figure 2A and 3C, lanes 1, 2, 3 & 5). Phospho-AKT levels were increased in PTEN-null U87 cells and U87 cells reconstituted with R130M and to lesser extent in U87 cells expressing the G129E mutant (Figure 3C, lane 1) as compared to the wild type PTEN transduced cells (Figure 3C, compare lanes 1, 2 & 4 to lanes 3 & 5). The pattern of phosphorylated AKT was similar comparing the

different U87 mutant expressing cell lines assayed *in vitro* or *in vivo* (compare Figure 2A to 3C). Despite these similarities, there was a dramatic difference in the growth of tumors derived from parental U87 cells compared to cells reconstituted with wild type PTEN (Figure 3A & B). The average volume of U87-derived tumors on day 25 after implantation was 848±203 mm^3, compared to 91±27 mm^3 for tumors derived from PTEN-reconstituted cells (n =5, p<.0001). From these data we conclude that the loss of inositol phospholipid phosphatase activity results in deregulated tumor growth comparable to the total ablation of catalytic activity (p>.1). From these data we can see that reconstitution with catalytically dead or PIP_3 deficient PTEN has a negative effect on tumor growth.

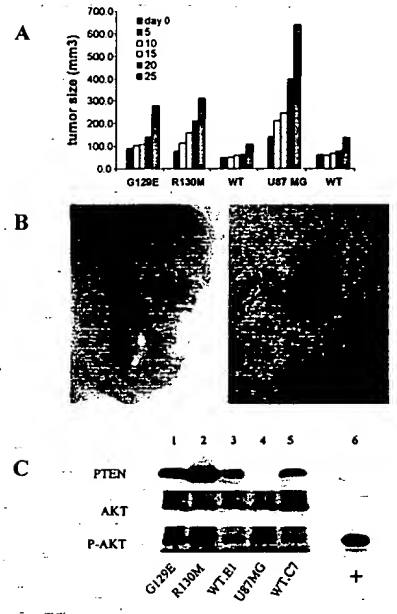

Figure 3. Effects of PTEN on growth of U87MG cells *in vivo*. (A) Cell growth*in vivo*. In order to determine the rate of cell growth *in vivo*, equal amount of cells ($5x10^6$) from each cell line were implanted at the right ventral flank by subcutaneous injection (see legend). The formation and growth of the subcutaneous tumor was monitored and the volume of the tumor was determined by a three dimensional measurement at the times indicated (day 0, the date of implantation, no tumor is detected). Data were analyzed by Student's t-test and differences were significant comparing the PTEN deficient (U87MG, R130M, G129E) to the wild type PTEN (WT.E1, WT.C7), n=5, number of mice p<.0001 B) Stereophotography of subcutaneous tumor sites in mice implanted with the parental U87 tumor, PTEN minus (left panel) versus wild type PTEN reconstituted tumor cells (right panel). These tumors represent 25 and 42 days after implantation for PTEN minus versus wild type PTEN reconstituted tumors, respectively. C) Immunoblot of cryostat tissue sections from subcutaneous tumor for the expression pattern of PTEN, AKT and phosphorylated AKT. Frozen tissue sections were solubilized in Laemmli sample buffer, total protein was quantitated and equal protein was loaded on SDS PAGE. The data shown are representative of tissue analysis from 5 animals per experimental group

PTEN controls tumor-induced angiogenesis.

To assess the angiogenic potential of the parental U87 cells compared to the cells reconstituted with wild type or mutant forms of PTEN, we stained cryostat sections from subcutaneous tumors for CD31 (PECAM). CD31 is an endothelial marker that is used to measure the microvessel density of these tumors. Microvessel density was assessed from multiple digitized images of CD31-stained tumor tissue at 100x magnification (3 fields were evaluated per tumor) and counted blindly for number of CD31 positive microvessels per unit surface area as described (36). Reconstitution of PTEN expression in U87 cells dramatically suppressed the angiogenic response *in vivo* (Figure 4A & B). Quantitation of microvessel density in tumors derived from parental U87 cells (77±13) and U87 cells expressing wild type PTEN (38±7) revealed an ~50% suppression of angiogenesis (Figure 4C)(n=5, p<.001). The microvessel density of tumors derived from U87 cells reconstituted with catalytically impaired PTEN (R130M, 84±15 or G129E, 69±16) were not significantly different (p>.05) from the parental U87 cell line (Figure 4C). The levels of phospho-AKT detected within the tumor mass *in vivo* suggest a mechanistic link between the loss of the inositol lipid phosphatase function of PTEN, the phosphorylation status AKT and the angiogenic phenotype within the tumor. Recent *in vitro* data suggest a link between PTEN and downstream targets including AKT, HIF1α and VEGF in the potential control of angiogenesis (37, 38).

We used the RNAase protection assay (RPA) to examine the effect of PTEN on thrombospondin 1 (TSP-1) expression. RPA was performed with a TSP-1 specific probe in U87MG cells constitutively expressing wild type PTEN or G129R PTEN (Figure 4D). The data demonstrate that wild type and not mutant PTEN expression activates TSP-1 in U87 cells. To confirm these results, we performed Western blot analysis for TSP-1 expression using a retroviral-based ecdysone-inducible PTEN expression system (39). Inducible and dose-dependent expression of PTEN was confirmed in U87 cells and we noted that the induced expression of wild type PTEN and not G129E PTEN resulted in augmented thrombospondin 1 expression (Figure 4E). Therefore, our data demonstrate that PTEN positively modulates the expression of thrombospondin 1, a negative regulator of angiogenesis (Figure 4D and E)(40, 41). These data suggest a potentially novel mechanism by which PTEN may regulate angiogenesis through the induction of TSP-1. It is noted that angiogenesis is not completely abrogated by PTEN replacement (Figure 4C) and that PTEN does not completely suppress levels of phosphoAKT within the tumor (Figure 3C). These data suggest that the capacity of PTEN to suppress AKT may be negatively regulated within the cell. The mechanism(s) responsible for the negative feedback control of PTEN is currently unknown (discussed in Aim 1). Importantly, our data provide the first direct evidence that PTEN controls the angiogenic behavior of tumor tissue *in vivo*. Finally we propose that PTEN may control the "angiogenic switch" an event required for tumor progression *in vivo*.

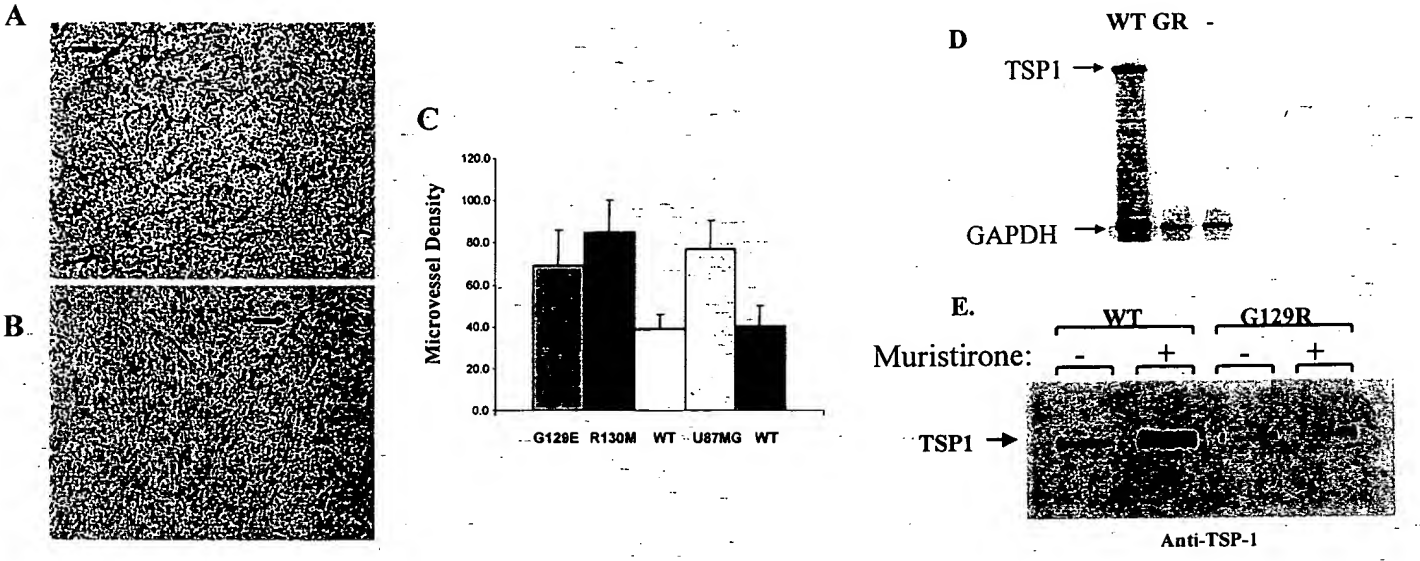

Figure 4. PTEN suppresses angiogenesis. Immunohistochemical analysis of staining with CD31 antibody to evaluate the angiogenesis response within the parental U87MG tumor (A) and PTEN reconstituted tumor (B), implanted into the subcutaneous tissue. In PTEN minus and tumors expressing mutants of PTEN, there are more new vessels formed (angiogenesis) (upper panel, arrow indicated) than in wild-type PTEN reconstituted tumor (lower panel), indicating the PTEN has direct influence on angiogenesis during tumor growth. C). Microvessel density (MVD) counts were performed on tumor tissue stained with anti-CD31 antibody as described (36) to determine effect of expression of PTEN and specific PTEN mutants (G129E or R130M) on tumor induced angiogenesis. Bars represent standard deviation, 5 animals per group. Statistical analysis by Student's t-test demonstrate significant difference between MVD of PTEN null and PTEN catalytic mutants as compared to wild type PTEN reconstituted tumors, n=5, number of mice p<.001. D). PTEN regulates the expression of thrombospondin-1 (TSP-1) in U87MG cells. RNAase protection assay was used to measure levels of TSP-1 mRNA in wild type PTEN expressing U87 cells or cells transduced with a mutant catalytically dead PTEN (G129R). U87MG cells were infected with retrovirus encoding wild type PTEN (WT), the catalytically dead, G129R mutant (GR) or empty vector retrovirus (-) and selected for 10 days in puromycin. RNA was harvested and RNAase protection assays were carried out using probes for TSP-1 and GAPDH (method section, d). A probe for glyceraldehyde phosphate dehydrogenase (GAPDH) was used as a normalization control. E) TSP1 immunoblot analysis. U87MG transduced with wild type PTEN (WT) or a catalytic mutant PTEN (G129E) in an ecdysone inducible expression system (39) were induced (48 hours) with 0.5 uM muristirone or assayed without induction to determine the effect of PTEN expression on the induction of TSP-1 by Western blotting. Cell lysates were prepared (two separate experiments, A and B), resolved on SDS-PAGE and probed with anti-TSP-1 antibody. HT1080 is a cell line with known high-level expression of TSP-1. There is clear up-regulation of TSP-1 in wild type transduced U87 cultures compared to U87 cells expressing the lipid phosphatase deficient G129R mutant PTEN, where no induction occurs.

Effect of PTEN on brain tumor related survival.

To determine whether the expression of the PTEN affects the survival of mice in this brain tumor model, U87 cells expressing either wild type or mutant forms of PTEN were implanted under stereotactic control into the right frontal lobe of nude mice (Figure 5B, see arrow for site of implantation). The results indicate that the reconstitution of wild type PTEN in U87 cells suppressed the malignant potential of these cells in our orthotopic animal model. Thus, there was 90% survival at 40 days in animals implanted with the wild type PTEN-reconstituted U87 cells compared to 100% mortality of mice implanted with the parental cells at 27 days (Figure 5C). PTEN reconstituted tumor cells were noted to grow more slowly when implanted in the frontal lobe (Figure 5, compare A & B) and remain circumscribed to that area of brain (data not shown). U87 cells reconstituted with mutants of PTEN, either ablated in inositol lipid phosphatase activity (G129E) or catalytically-inactive (R130M), displayed a phenotype similar to the PTEN-negative, parental U87 cells (Figure 4C). Animals with tumors derived from U87 cells reconstituted with PTEN-G129E displayed slightly prolonged survival (50% at day 30) compared to those implanted with parental U87 cells, however, all animals died by day 40. These data suggest that ablation of the inositol lipid phosphatase activity of PTEN alone is sufficient to yield an angiogenic (Figure 4) and malignant glioma (Figure 5C) following implantation of U87 tumor cells.

The data demonstrate that mutations in the PTEN tumor suppressor in U87 glioblastoma cells are an important determinant of their malignant behavior *in vivo*. The data indicate that the effects of PTEN are exerted at the level of tumor angiogenesis *in vivo*. PTEN was first characterized as a dual specificity phosphatase that displayed selectivity for acidic substrates, including both protein and non-protein targets. Therefore, a question remains as to whether PTEN dephosphorylates protein substrates *in vivo*. There have been suggestions that a primary target of PTEN may be the protein tyrosine kinase FAK, which is a regulator of integrin-dependent signaling and cell migration (42). However, the properties of U87 cells reconstituted with the PTEN-G129E mutant suggest that it is the lipid phosphatase activity of PTEN that is a critical determinant of its function in this brain tumor model. Holland et al recently reported that the introduction of activated AKT and Ras into glial cells of the mouse brain results in the development of glioblastomas (43). Thus, our data suggest a potential therapeutic benefit of inhibitors of PI-3 kinase and downstream targets such as AKT in treatment of malignant gliomas. This brain tumor model will be useful for the identification of potential physiological targets that are regulated by PTEN (TSP1) and will allow us to define the mechanism by which PTEN controls angiogenesis as described below in specific aims 1-3.

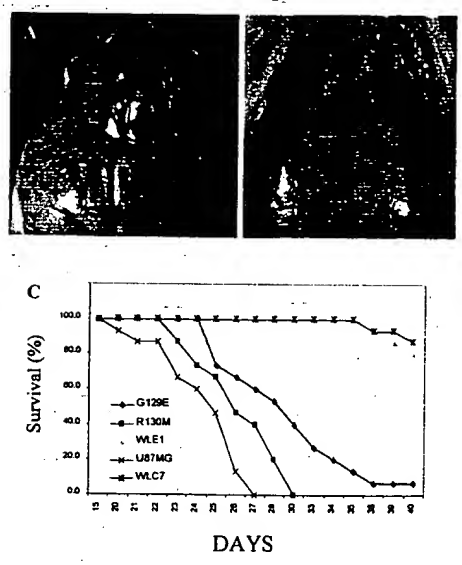

Figure 5. Effects of PTEN reconstitution on survival in an orthotopic brain tumor model. Equivalent number of parental U87 cells or U87 cells reconstituted with wild type or mutant alleles of PTEN (see legend)(1 x 10^6 cells) were implanted in right frontal lobe of nude mice. Cells were cultured in fresh medium for 24 hours and harvested, adjusting the cell concentration to 1 x 10^6 in 10 ul of RPMI medium. Mice, general anesthesia were placed into the stereotactic device (model 963, Kopf, Tugunga, CA). Stereotactically controlled drill assembly was used to provide a 0.3 mm deep and 0.8 mm/diameter hole in cranium at a position 0.5 mm anterior and 1.2 mm lateral to the bregmal anatomical landmark. Tumor cells (1 x 10^6) were introduced slowly through a 10 ul Hamilton syringe at a depth of 2.5 mm at a rate of 2 ul per minute. We then slowly removed the needle at a rate of 0.5 mm/min. After needle removal we seal the hole with bone wax and close incision with wound clip. Stereophotography of whole brains from mice implanted with U87MG tumor cells (day 25) (A), or PTEN reconstituted (day 42) (B). The implantation site is shown by position of arrow in the wild type PTEN reconstituted tumor, (B) (magnification x 20). C) Survival plots for mice implanted with PTEN minus or parental U87 cells transduced with mutants of PTEN as shown. Survival data represents 15 animals per experimental group. n=15, $p < .0001$ for difference observed between the PTEN + and PTEN – groups for survival.

Manuscripts supported during this funding period 7-1-97 to 7-30-2000 (Appendix)

Erdreich-Epstein, A., Liu, M. Liu, Y.B. and **Durden, D. L.** Protein tyrosine phosphatase inhibitors in FcγRI-induced myeloid oxidant signal transduction. *Exp. Cell Res.* 237: 288-295, 1997 .

Park, R.K., Liu, Y.B., Kyono, W., and **Durden, D.L.** CBL-GRB2 adapter protein interaction in immunoreceptor tyrosine activation motif (ITAM) signaling. *J. Immunol.*, 160(10):5018-5027, 1998.

Chu, J., Liu, Y, Koretzky, G.A. and **Durden, D.L.** SLP-76-CBL-Grb2-Shc interactions in FcγRI signaling. *Blood*, 92(5):1697-1706, 1998.

Kyono, W. T., De Jong, R., Park, R. K., Liu, Y.B., Heisterkamp, N., Groffen, J. and **Durden, D.L.** Differential interaction of Crkl with Cbl or C3G, Hef-1 and γ-subunit ITAM in myeloid FcγRI signaling. *J. Immunol.*, 161:5555-5563,1998 .

Izadi, K., Erdreich-Epstein, A., Liu, Y., and **Durden, D.L.** Characterization of Cbl-Nck and Nck-Pak1 interactions in myeloid FcγRII signaling. *Exp Cell Res.*, 245:330-342, 1998.

Erdreich-Epstein, A., Liu, M. Kant, A., Izadi, K., Nolta, J. and **Durden, D.L.** CBL functions downstream of Src kinases in FcγRI signaling in primary human macrophages *J. Leuk. Biol.*, 65:523-534, 1999.

Park, Rae-Kil, Izadi, K., Deo, Y.M., Liu, YB and **Durden, D.L.** Role of Src in the modulation of multiple adaptor proteins in FcαRI oxidant signaling. *Blood*, 94: 2112-2120,1999.

Park, R.K., Erdreich-Epstein, A., Liu, Y.B., Liu, M., and **Durden, D.L.** FcγRI receptor modulates the SHC-GRB2-CBL interaction and augments the membrane SHC-GRB2-SOS complex to activate RAS. *J. Immunol.*, 163: 6023-6034, 1999.

Erdreich-Epstein, A., Shimada, H., Groshen, S., Liu, M., Metelitsa, L., Kim, K.S., Stins, M., Seeger,R.C. and **Durden, D.L.** Integrins αvβ3 and αvβ5 are expressed by endothelium of high-risk neuroblastoma and their inhibition is associated with increased endogenous ceramide. *Cancer Research*, 60: 712-721, 2000.

Wen, S., Stolarov, J.,Myers, M.P., Su, J.D., Wigler, M.H., Tonks, N.K. and **Durden, D.L.** PTEN controls angiogenesis and growth of malignant gliomas. (Submitted), Nature Genetics, 2000.

Research Plan.

Summary: We have established in an orthotopic brain tumor model, that PTEN exerts control over the angiogenic process, induced by high-grade glioma tumor cells (Figure 2-5). PTEN also controls growth of these intracranial tumors and survival in mice. Many questions remain; how does PTEN control angiogenesis in malignant brain tumors? If PTEN controls angiogenesis, will this control growth? if growth is controlled, will PTEN replacement or inhibition of PI-3 kinase decrease mortality? We will primarily focus on how PTEN controls angiogenesis. Our examination of tumor growth and animal survival will be aimed at correlating PTEN effects on angiogenesis with tumor growth and animal survival (as described in Figure 3-5 of Preliminary Data).

Aim 1. Mutations in PTEN have been implicated in the malignant progression of adult and pediatric glial tumors (1, 2). We will use our orthotopic nude mouse brain tumor model to determine the role of PTEN and specific mutations in PTEN in angiogenic response of malignant gliomas. We will generate mutations in PTEN, stable clones of glioma tumor cells expressing these mutants and test these cells in our orthotopic nude mouse model for effects on angiogenesis, tumor growth and animal survival. We will explore the mechanism by which PTEN regulates thrombospondin 1 expression in the control of angiogenesis. We will examine the effect of PTEN on the MDM2/p53 signaling axis.

Aim1. Role of PTEN in the regulation of angiogenesis.

TSP-1, an established inhibitor of angiogenesis.

Rationale. As mentioned above, we demonstrated that PTEN controls the expression and secretion of TSP1 (Figure 4D and E). The elegant work of several laboratories, Lawler et al and Bouck et al have clearly established that TSP1 exerts a negative control over endothelial cell physiology and suppresses angiogenesis (41, 44). A considerable body of literature has established which domains of this complex matrix protein exert its antiangiogenic effects and worked out some of the mechanisms which underlie TSP1 affects on endothelial cell survival and angiogenesis (45). TSP1 undergoes a complex set of interactions with a number of other proteins in mammalian systems including: 1) TSP1 binds to latent TGFβ1 and participate in the control of TGFβ1 activity *in vivo* (46). 2) TSP1 interacts with CD36 on endothelial cells (47) and induce apoptosis in these cells (45) 3) TSP1 can interact with the vitronectin receptor αvβ3 and integrin associated protein (CD47)(48, 49). Most of these interactions are poorly understood but likely contribute to regulation of endothelial differentiation and survival required for formation of blood supply within the tumor (50). Moreover angiogenesis is a complex process involving numerous cell surface receptor and intracytoplasmic and intranuclear signaling events (28, 51).

We will design experiments to examine how PTEN regulates TSP1 and determine if the observed effect of PTEN on TSP1 expression (Figure 4D and E) is responsible for suppression of angiogenesis, growth and animal survival in PTEN reconstituted glioma cells *in vivo* as described above in preliminary data (Figure 3-5). We will not design experiments to examine how TSP1 may regulate endothelial physiology or angiogenesis (this is beyond the scope of our proposal).

How does PTEN control angiogenesis and TSP1 expression?

Experiments. Despite significant progress, the mechanism(s) by which PTEN acts as tumor suppressor is not well understood at this time. An increasing number of mutations of PTEN are being defined in patients with cancer involving numerous organs (prostate, endometrium, breast, thyroid, bladder, lymphoma/leukemias and brain). As we continue to catalog these mutants we begin to assemble useful information regarding role of PTEN domains in tumor suppressor function (25, 52). The C terminal PDZ domain of PTEN was recently observed to functionally interact with a PDZ-containing membrane scaffolding protein, MAGI-2 a likely control point for membrane targeting. Several tumors have been described with mutations which truncate this PDZ domain (25). This would lead to a loss of function of PTEN. Many PTEN mutations occur in the catalytic domain suggesting that the catalytic activity of PTEN is important for tumor suppression.

Mutants of PTEN. In this aim we will generate two classes of PTEN mutants; 1) site directed mutations within the catalytic domain (N terminus) (Figure 6) and 2) C terminal mutations which will alter membrane localization and protein stability. We will express these mutants in U87, U373 and U251 glioma cells to determine the role of PTEN in glioma-induced angiogenesis. We will also express these mutants in T98G glioma cells which is p53 deficient to compare effect of PTEN on TSP1 in absence of p53. In addition to the mutants shown in Figure 1-4 (G129E, R130M), we will mutate cysteine, at position 124 to serine to create a catalytically dead phosphatase substrate trap mutant which is predicted to block the capacity of the endogenous PTEN to dephosphorylate substrates by binding to these substrates with high affinity. This would be expected to augment the phosphorylation of PI at the D3 position in cells expressing wild type PTEN. The C124S mutant has been observed to augment phosphorylation of substrates and activate AKT signaling pathways (53). Mutant expressing cell lines will be studied extensively as described above in Figures 2-5 and in more detail as relates to control of TSP1 and p53 (described below).

The C-terminus of PTEN has been implicated in control of protein stability, membrane localization and catalytic activity at least partly through the action of phosphorylations occurring with the C-terminal tail

region (Figure 1). The C-terminal tail (composed of approximately 50 amino acids) has recently been reported to undergo phosphorylation of serine/threonine residues (S380, T382 and T383) (27). Phosphorylation of these sites has been shown to regulate protein stability and phosphatase activity suggesting a mechanism by which phosphorylation may regulate PTEN function (27). Other data suggest that phosphorylation of the PDZ domain may regulate interactions with other PDZ domains suggesting a possible mechanism for the negative regulation of PTEN (54).

We will generate an epitope-tagged PTEN protein with altered membrane localization and/or protein stability. The wild type PTEN will be engineered without the PDZ domain (5 amino acids in extreme C-terminal tail region (delta-PDZ) or the PDZ domain will be replaced by a consensus farnesylation CaaX site as described (55). We will perform pulse chase experiments to determine protein stability of these C-terminal truncation and point mutants as described (27). We will examine the phosphatase activity of these mutants *in vitro* against PIP_3 and acidic peptide substrates as described by Myers et al (13). Importantly, we will use antibodies specific for the epitope-tag (HA) to immunoprecipitate mutant PTEN protein from tumor tissue expressing these mutants in order to examine the biochemical activity of these PTEN mutants *in situ*. The effect of these mutations will be to do away with the PDZ membrane localization signal or in the case of CAAX.PTEN, create a constituitive membrane localizing signal. Each mutant will be epitope-tagged in the N-terminus with hemagglutinin (HA)(YPDTVPDYA) to allow for confocal imaging of the mutant and specific immunoprecipitation of mutant in cells which have wild type PTEN expression. It is possible that the subcellular location of PTEN mutants may affect their activity *in vivo*. This has been shown to be the case for PI-3 kinase and AKT kinases (discussed in Aim 2) (56, 57).

The mutagenesis and subcloning strategy will be stratified as follows: 1) first we will make the wild type and all site-directed mutants of PTEN as HA-epitope tagged constructs as described (G129E, R130M, C124S and the triple mutant, S380A/T382A/T383A, (60% of mutants are made at this time)(Figures 1-5)(Figure 6). 2) Delete the PDZ domains from the wild type PTEN using PCR primers which will amplify the HA sequences in the N terminus and amplify PTEN starting at position of 399 within the C terminus. 3) Introduce by PCR amplification, the RAS CaaX farnesylation sequences into C terminus to replace the PDZ domain of wild type PTEN with farnesylation site. 4) If necessary, we will also introduce into the PTEN N-terminus a SRC kinase myristorylation site. All will be engineered by PCR to contain a methionine start site surrounded by a Kozak consensus sequence and a stop codon if C terminal change. In stratum 4, it will be necessary to place the HA epitope tag in the C terminus of PTEN, again using the PCR primers to introduce the MYR sequences into the PTEN N-terminus (not shown). All constructs will be sequenced following the final ligation into expression plasmids to assure fidelity.

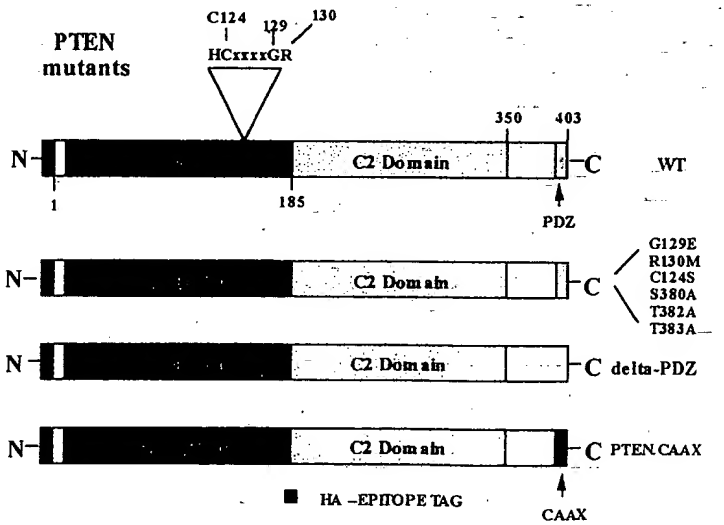

Figure 6. Schematic representation of PTEN mutations planned for Specific Aim 1.

Evaluation of PTEN Mutants. The purpose of generating specific mutations within the catalytic domain has been discussed above. Many point (missense) mutations have been observed in human tumor tissue localized to the N terminal catalytic region of PTEN (58). Loss of catalytic activity is associated with tumor progression in our animal model (Figure 2-5) and in human cancer patients. Therefore we will define the effects of these point mutations in the process of tumor-induced angiogenesis. Importantly the N terminus and C terminus of PTEN may interact as part of normal physiologic regulation of this phosphatase (59). Currently, very little is known regarding the physiologic positive and negative regulation of PTEN in the cell. Recent data from Vasquez et al defined a function for the C terminal phosphorylation sites in PTEN in the regulation of phosphatase activity and protein stability (27)(Figure 8). They suggest that the phosphorylation of PTEN serves to negatively regulate the phosphatase activity. Upon dephosphorylation, PTEN is activated and simultaneously degraded. This suggests a high degree of regulation of PTEN activity which could affect the capacity of PTEN to control tumor induced angiogenesis. We will therefore examine the wild type PTEN in the context of C terminal mutations for effects on angiogenesis (S380A/T382A/T383A, delta-PDZ-PTEN or CAAX.PTEN). We will express these mutants in glioma cell lines and examine effects of these mutants on angiogenesis, tumor growth and animal survival after intracranial implantation. We will evaluate effects of these mutants on downstream signaling events implicated in the control of TSP1 expression i.e. MDM2/p53 dependent transcriptional activity (as defined below).

In 50% of human tumors, mutations in PTEN result in nonsense and premature truncation of PTEN protein (25, 58), these truncations occur in more C terminal region of molecule suggesting a role of C terminus in PTENs control of tumor growth. This concept is further supported by the observation that removal of the PDZ activates the phosphatase activity of PTEN. We hypothesize that the C terminus of PTEN may control important aspects of angiogenesis via either phosphorylation related regulatory changes in catalytic activity or subcellular localization. By crystal structure analysis it is clear that the catalytic domain is in close contact with the C terminal C2 domain suggesting some kind of cooperative catalytic interaction (25, 60). This aspect of PTEN function and regulation has not been explored to date. Hence we will design mutants with altered subcellular localization and determine the effects of these mutations on the capacity of glioma cells to induce the process of angiogenesis. For example, if we take a wild type PTEN construct which potently suppresses angiogenesis (Figure 4A-C), tumor growth (Figure 3) and promotes animal survival (Figure 5) and mutate the PDZ domain to decrease membrane localization what will this do to its capacity to act as tumor suppressor? We will compare the effects of these PDZ mutants on angiogenesis to the capacity of these mutants to affect the induction of p53 transcription and TSP1 synthesis as described below. We will examine effects of these mutants on levels of phosphoAKT in U87, U373 and U251 glioma cells *in vitro* and *in vivo* as described in Figure 3A-C. We will perform experiments to establish the subcellular location of these delta-PDZ mutants compared with the wild type PTEN as described previously in our laboratory (see reference #3, appendix for detail methods, subcellular fractionation) (61).

The purpose of the PDZ domain deletion will be to examine the effect of loss of PTENs membrane localization on angiogenesis, TSP1, tumor growth and animal survival. The CAAX farnesylation site or Src kinase myristorylation site will constitutively localize wild type PTEN or mutants to the membrane. Recent data suggest that the extreme C terminus of PTEN is phosphorylated on serine and threonine residues (27) (S380, T382, T383)(Figure 6) raising the possibility that the PDZ interaction with MAGI-2 (a scaffolding protein)(26) may be regulated within the cell by proximal phosphorylation events. Leslie et al described a mutation which deleted the PTEN PDZ domain (delta-PDZ) (62). This mutant was expression in U87 and NIH 3T3 cells. The results revealed a requirement for the PDZ domain for the regulation of PDGF induced membrane ruffling but not for the control AKT phosphorylation. The PDZ mutant was as deficient as catalytically dead mutants (C124S) in the control of membrane cytoskeletal reorganization. The PDZ mutant suppressed the activation of AKT whereas catalytically dead PTEN mutants (G129E,R130M) are deficient in both activities. We interpret this data to suggest that the PDZ interaction is important for cytoskeletal interaction relating to PTEN function. Other data suggest the PDZ domain may regulate certain components of PTEN activity and contribute to tumor progression.

Currently the function of PDZ or the C terminal phosphorylation sites in PTEN as relates to the control of AKT, PIP_3 or angiogenesis are not known. Data from the PDZ deletion mutant point to a division of function as relates to interaction with the scaffolding protein, MAGI-2 which could be important for control of angiogenesis and tumor progression. These investigators did not look at membrane localization or any biologic function of PTEN. We have shown that U87 cells devoid of PTEN have very high RAC-GTP levels and that PTEN reconstitution suppresses the activation of RAC. Other data from our laboratory demonstrate the PTEN suppresses motility of U87 tumor cells on vitronectin. The capacity of PTEN to control RAC, likely through an effect upon PIP_3 may also contribute to the capacity of PTEN to suppress angiogenesis. If as reported by Leslie et al the PDZ mutant maintains its activity towards AKT but does not suppress RAC as would be expected, it will be interesting to examine the effect of PDZ deletion on angiogenesis and tumor growth. Accordingly we will examine the effect of PDZ mutation on cellular RAC activity using the PAK-PBD pull-down assay to detect GTP-RAC levels in cells expressing the different PTEN mutants (63).

Farnesylation or myristoylation of PTEN would permanently place the PTEN mutants within the membrane in the proximity to the Src/FAK kinases or GTPase specific sites, respectively. The mutants will be epitope-tagged with HA or FLAG so their location within the cell can be tracked by confocal microscopy. We will use biochemical subcellular fractionation methods previously established in our laboratory to examine the subcellular localization of all PTEN mutants as compared to the wild type PTEN (61, 64). We will carry out this analysis in cells growing in tissue culture and in glioma cells growing within a tumor mass. The epitope tagging of PTEN mutants will allow us to examine tumor cell specific localization of PTEN *in vivo*, i.e. PTENs subcellular localization within a tumor mass. Once stable clones of cells are selected expressing these mutants we will evaluate the effects of these mutants on angiogenesis and phosphorylation status of AKT *in vitro* and in the tumor as defined in Figures 2-5 above. Anti-HA immunoblot analysis will be used to determine level of expression of mutants and wild type PTEN in U87, U373 and U251 glial cells. Several clones of each mutant will be isolated with different levels of HA-PTEN expression in an effort to correlate levels of expression to phenotypic effects of the mutant on angiogenesis, tumor growth and animal survival. Additional analysis will be performed of all mutants for effects on TSP1 expression and the postulated regulatory pathways that control TSP1 transcription (p53 transcription)(see below for details on TSP1 and p53 experiments). We will examine effects of PTEN mutant expression on glioma cell lines *in vitro* and in our animal model for microvessel density, tumor growth and animal survival after intracranial implantation as described in methods section, **b** (Figure 5). We will correlate these findings with biochemical analysis of phosphatase activity of each PTEN mutant within glioma cells grown *in vitro* and *in vivo*.

Does thrombospondin control angiogenesis and growth in our orthotopic model? Our RNAase protection assay and Western blot analysis demonstrate that PTEN controls the expression of TSP1 in glioma cells grown in tissue culture (Figure 4D and E). To determine if this process occurs in the tumor we will utilize immunohistochemical methods to examine the levels of thrombospondin in tumor tissues as defined in Figure 3C. We will use Western blot analysis to evaluate TSP1 expression in tumor tissue from PTEN null and PTEN wild type and mutant reconstituted tumors to determine if wild type PTEN reconstitution turns on TSP1 expression. We will ask whether TSP1 expression correlates with decreased angiogenesis as described in Figure 4A-C. These data will not prove cause and effect as it relates to TSP1 control of angiogenesis. To address this question, we will prepare stable clones of the parental U87, U373 and U251 glial cell lines expressing the full length TSP1 cDNA and test them for angiogenic and growth capacity in our subcutaneous and orthotopic brain tumor model. The TSP1 cDNA will be subcloned into the pBABEpuro retrovirus already shown to effectively express the PTEN gene in U87 and U373 cells (Figures 2-5)(data not shown). Stable clones will be selected in puromycin and injected as described above into the frontal lobe or subcutaneous tissue. Western blotting as described (Figure 4E) will confirm TSP1 expression in these clones. Angiogenesis will be assessed by a determination of microvessel density by CD31 staining as shown in Figure 49A-C and by monitoring the growth of tumor (Figure 3A). Effects of glial cells expressing TSP1 on mortality after orthotopic brain injection will be assessed as described in Figure 4. Final confirmation of TSP1 expression will require immunohistochemical analysis of tumor tissue from TSP1 cDNA transduced cells versus empty vector controls. As noted in Aims 2 and

3 we will carry this analysis of TSP1 throughout this proposal confirm that the PTEN/AKT axis controls TSP1 expression.

We will express the wild type TSP1 protein in U87, U373, U251 and T98G cells constitutively and determine whether TSP1 expression exerts an effect on angiogenesis. These combined data will allow us to determine if TSP1 controls angiogenesis in our model. We will examine effects of TSP1 expression on tumor growth rate and animal survival in an effort to determine again whether TSP1 will reduce tumor progression as has been observed in other systems.

Does PTEN exert an effect upon p53 transcription? PTEN controls TSP1 expression and Dameron et al reported that TSP1 expression is regulated by p53 mediated transcriptional activity (65). Currently there are no reports demonstrating a direct effect of PTEN or AKT on p53 transcription. Recent evidence has implicated the PIP_3/AKT cascade in the regulation of a number of downstream transcriptional events. e.g. control of NFKB activation is controlled by AKT (66). The Forkhead (FH) transcriptional control of FAS expression is negatively regulated by AKT phosphorylation of FH which excludes FH from the nuclear compartment (67, 68). PTEN/AKT has been implicated in the control of HIF1α transcription an effect which could affect the expression of VEGF (37, 38). Thrombospondin expression is regulated by p53 transcription a result which led us to hypothesize that PTEN may regulate p53 activity. One important negative regulator of p53 activity is MDM2 (69). AKT activation is known to prevent apoptosis a process which in many cells is mediated by p53 (69). These data taken together suggested a possible connection between PTEN/AKT and p53. Preliminary data generated in collaboration with Dr. David Donner, IU School of Medicine (see letter of collaboration) suggests that MDM2 is phosphorylated by AKT and that this phosphorylation event induces the movement of MDM2 from the cytoplasm into the nucleus where it is associated with the degradation of p53. Hence the activation of AKT results in reduced levels of p53. Conversely, the inhibition of AKT by PTEN would be expected to reduce the levels of MDM2 and lead to increased p53. If true, this hypothesis could account for the effect of PTEN on p53 and subsequently TSP1 expression (see schematic Figure 7). To determine the effects of PTEN on p53 we will take advantage of stable U87 cell lines expressing wild type PTEN or mutants of PTEN. Importantly, the U87, U373 and U251 cells are wild type for p53 expression. Preliminary data from our laboratory suggest that PTEN/AKT controls MDM2 phosphorylation and this phosphorylation controls p53 degradation, levels of p53 within the nucleus and p53 transcriptional activity. This is the first clear demonstration of connection between PTEN and p53 pathways and would suggest a mechanism by which PTEN could control TSP1 expression within a brain tumor.

To address this question, we will examine the effect of PTEN reconstitution on p53 transcription using a p53 response element linked to a luciferase reporter. U87 glioma cells will be transiently transfected with pGL2 plasmid containing MDM2luc which is composed of exon1-intron1-exon2-intron2-exon3-TATA of the MDM2 promoter linked to firefly luciferase. Exon 1 of MDM2 promoter contains two p53 responsive elements. A control plasmid designated MDM2pvLuc contains exon-2-intron2-exon3 is missing the p53 response elements will be used to assess p53 specificity of this assay. The pCMVβgal is cotransfected with the luciferase reporter to normalize for transfection efficiency. The transfection protocol used in Tropix and Galacto-light kit for β galactosidase. The luciferase assay system used is from Promega. We will compare the p53 dependent luciferase promoter induction in U87, U373 and U251 cells reconstituted with wild type PTEN or the G129E or R130M catalytic mutants. Finally, we will perform laser confocal imaging of cytoplasmic and nuclear PTEN, p53 and MDM2. We will also perform biochemical analysis of p53 and MDM2 levels in U87 glioma cells growing in tissue culture and as tumor mass. We will fractionate cell lysates into a nuclear and nonnuclear preparation to examine the relative expression of p53 andMDM2 in these compartments. We will examine the nuclear levels of p53 in glioma cells in vitro and in vivo as described (Figure 3C). If positive results are obtained from our analysis of PTEN effect on p53, we will procure the TSP1 promoter as described to examine the effect of PTEN reconstitution on TSP1 specific gene expression (65) (Noel Bouck, Northwestern University, Chicago. IL). These data will provide conclusive evidence that PTEN exerts control over p53 transcription of TSP1 in glioma cells.

Anticipated Results. The control of angiogenesis is complex with contribution from numerous positive and negative regulatory molecules. Our preliminary data establish the first direct evidence that PTEN reconstitution can negatively modulate the tumor-induced angiogenic response (Figure 4A-C).

PTEN mutants. Our preliminary data suggest that the capacity of PTEN to suppress angiogenesis is dependent upon PIP_3 phosphatase activity. We will continue our analysis of the G129E and R130M PTEN constructs in additional glioma cell lines (U373, U251 and T98G). We would predict that if the capacity to suppress angiogenesis is dependent on p53 transcription of TSP1 that the U87, U373 and U251 cell lines reconstituted with wild type PTEN will have suppressed TSP1 and angiogenic activity. Whereas the T98G cell line (deficient in p53) reconstituted with PTEN would not induce p53 transcription of TSP1 and PTEN would not suppress the angiogenic response. To confirm this is dependent on p53 we will need to express p53 in the T98G cell line and show that we can reconstitute the PTEN-p53 signaling axis.

Based on the effect of PDZ deletion on the regulation of AKT, we anticipate that removal of PDZ domain will not affect PTENs suppressive effects upon angiogenesis. This prediction is based on concept that PTEN may not require the PDZ domain to mediate its PIP_3 phosphatase activity. On the other hand, a CAAX.PTEN or myristoylated wild type PTEN would be expected to have greater antiangiogenic activity than the wild type PTEN itself. Point mutations in PTEN which abrogate lipid (PIP_3) phosphatase activity without an effect on protein phosphatase function will allow us to dissect the effects of PTEN on PIP_3 alone versus PIP_3 effects combined with dephosphorylation of proteins (e.g. AKT, FAK, SHC, SRC, etc.). Currently there are no data which have shown *in vivo* evidence that PTEN controls levels of phosphorylation of specific proteins. Hence these mutants will allow us to determine effects of PI versus protein phosphatase on specific substrates *in vivo*. To this end, we anticipate that PTEN controls angiogenesis mostly through an effect of PIP_3 action on AKT since our data suggest that AKT controls levels of p53 (discussed further below).

The results from the expression of the triple phosphorylation site PTEN mutant (S380A,T382A,T383A) will be more difficult to predict since it is not clear whether these phosphorylations play a negative or positive role in PTEN function (27) (Figure 8, appendix). It is also possible that these phosphorylations mediate a state of dynamics for PTEN required for both phosphatase action and regulation. Based on current literature, we would suggest that this mutant would have increased phosphatase activity, decreased protein stability and less stable interaction with the membrane via the PDZ (Figure 8). We view this phosphorylation as a negative feedback loop for the control of PTEN action on PIP_3. Accordingly our model predicts that the expression of this mutant as compared to the wild type PTEN will more significantly suppress PIP_3 and phosphoAKT within the glial cells. Hence we predict this mutant would have a greater capacity to suppress angiogenesis and would increase p53 transcription and TSP1 expression *in vitro* and *in vivo*. We observe in our animal model that the level of phosphoAKT are not completely suppressed and some level of angiogenesis continues in wild type PTEN expressing tumors *in vivo* (Figure 3 and 4). We postulate that this phenomenon relates to a normal homeostatic control of PTEN through phosphorylation of the C terminus. If our idea is correct, the phosphorylation site defective mutant will be less anchored to the plasma membrane via the PDZ domain and serine/threonine dephosphorylation events which would normally anchor PTEN to the membrane would not be operable. Similarly, C terminal truncations of PTEN distal to the C2 domain have been observed in human tumors; these mutations would also disengage PTEN from its site of action, the membrane, leading to tumor progression and loss of control of angiogenesis. In contrast to this idea is data from the PDZ deletion mutant showing equal capacity to regulate phosphoAKT as compared to wild type PTEN (62). Hence we predict that both the phosphorylation site mutant and the PDZ deletion mutant will suppress angiogenesis when expressed in glioma cells. We will correlate the effects of these mutants on tumor growth, angiogenesis and animal survival with levels of p53, p53 transcription, TSP1 expression, phosphoAKT and RAC-GTP levels.

PTEN, p53 and TSP1. We predict that PTEN via its control over AKT exerts control over angiogenesis at least partly through the control of the anti-angiogenic matrix protein, TSP1. Based on preliminary data

from our laboratory we think that the control of TSP1 is exerted at the level of p53 through effects of MDM2 phosphorylation by AKT. Dr. David Donner, (see letter of collaboration) has mapped two phosphorylation sites in MDM2 (S166, S178) which are phosphorylated by AKT. The phosphorylation of MDM2 is associated with translocation of MDM2 into the nucleus and decreased levels of p53 in the nucleus. The affects of activated AKT on MDM2 /p53 axis may explain a potential mechanism for control of TSP1 and angiogenesis by PTEN. Herein, we will investigate the link between MDM2 phosphorylation, PTEN and TSP1 expression and tumor induced angiogenesis. First, we will determine how PTEN affects the phosphorylation of MDM2 in glial cells. Second, we will determine how different PTEN mutants impact on MDM2 phosphorylation and p53 levels. If PTEN reconstitution suppresses MDM2 phosphorylation and this correlates with increased p53 expression, the p53-TSP1 link would have been made. If this link exists we expect to see an effect of PTEN reconstitution of p53 nuclear translocation by immunofluorescence analysis. Further proof of concept would involve the overexpression of phosphorylation defective mutant of MDM2 in glioma cells. This would be expected to mimic effect of PTEN reconstitution. Likewise in Aim 2 we will determine the effect of activated and dominant negative AKT and PI-3 kinase mutants on these p53 signaling pathways including the control of p53 transcription and TSP1 expression (discussed below).

Alternatively, PTEN could control TSP1 through some other mechanism. Also the control of angiogenesis induced by PTEN reconstitution could be induced by another AKT dependent mechanism e.g. HIF1α is controlled by AKT pathway and HIF1α is known to control the production of VEGF a potent inducer of angiogenesis. It is possible that pro-angiogenic events are suppressed by PTEN e.g. VEGF, bFGF. We will evaluate the expression of these factors by performing immunoblots on tumor cells growing *in vitro* and analyze tumor tissue lysates as shown in Figure 3C. bFGF has been implicated in the transcription control of MDM2 and bFGF is a potent inducer of angiogenesis(70). In this case in order to avoid futile cycle, the effect of PTEN would be to suppress AKT and thereby inhibit HIF1α transcription and VEGF production. Semenza reported that p53 degradation is controlled by HIF1α and it is known that HIF1α is controlled by AKT pathways. The mechanism by which AKT controls HIF1α induced activation of VEGF is not understood. It is intriguing to suggest that in our model the HIFα/VEGF and bFGF secretory state of the tumor could also be controlled by PTEN via AKT effect on MDM2/p53 axis. This would make sense if one considers design of angiogenic and anti-angiogenic controls would avoid futile cycles to preserve cell economy. Accordingly, we will examine the expression of VEGF and bFGF in our glioma cells transduced with PTEN, AKT and PI-3 kinase constructs.

If our hypothesis for p53 regulation of TSP1 is not correct we will proceed to evaluate other AKT controlled transcriptional systems including HIF1α, Forkhead, NFKB etc. which could regulate the thrombospondin promoter. Currently, there is no data in the literature to support a role for these transcription factors in the control of TSP1 expression. Any observation along these lines would be unexpected and would generate new hypotheses. We will pursue first the most likely regulator of TSP1 promoter activity mechanisms involving p53. Particularly since we have evidence that PTEN regulates p53 via MDM2 phosphorylation. Our preliminary data supports an effect of AKT upon MDM2 regulation of p53; p53 would then regulate TSP1 expression.

TSP1 induction may not be causally linked to the inhibition of angiogenic activity. We can say that TSP1 is regulated by PTEN (Figure 4D & E) but we have no proof that TSP1 exerts an inhibitory effect on glioma-induced angiogenesis. To address this point we will express TSP1 off of a constitutive promoter in U87MG cells and implant these tumor cells intracranially as described to determine angiogenesis and survival pattern of tumor cells secreting TSP1. If positive data is obtained, this would provide evidence that TSP1 controls brain tumor induced angiogenesis and link our observation that PTEN controls TSP1 to effect on angiogenesis. It is likely that in order to avoid futile cycle of angiogenic and antiangiogenic regulators that angiogenic molecules may be controlled by some centralized regulatory network. It is possible that the central processing event for the "angiogenic switch" may involve AKTs control over MDM2/p53, so increased MDM2 yields decreased p53 which results in the induction of HIFα/VEGF and bFGF and the suppression of TSP1.

Potential problems and pitfalls. The orthotopic injection of human tumor cells into the nude mouse brain may not completely reflect the complex biology of human brain tumor initiation and progression. An advantage of our model is that 100% of the tumor cells will express the transgene i.e. mutants of PTEN, AKT and/or PI-3 kinase, allowing for *in vivo* biochemical analysis of tumor tissue (Figure 3C). As defined in Aim 3, we will use immunohistochemistry to evaluate human glioma tissue for PTEN and AKT status and compare these to results obtained in our the orthotopic model (Figure 3-5) which are positive or negative for PTEN. If disparity exists between our animal model and human tissue analysis, other animal models including the model recently described by Holland et al (43) will be considered. Many of these vectors used in this system are already in use in our laboratory and we are familiar with stereotactic brain injections (Figure 5).

The complexity of oncogene and suppressor oncoprotein interactions in human cancer dictate caution in interpreting data generated from animal models or in tumor cell lines. Despite this caveat, through careful selection of specific glial cell lines we hope to gain useful information about PTEN, PI-3 kinase and AKT function in glioma-induced angiogenesis. We will examine the effect of PTEN expression in multiple brain tumor cell lines. We will express mutants of PTEN in U87MG, U373MG, U251MG cells. We will also examine the effect of PTEN in the T98G glioma cell line which is deficient in p53 and lacks PTEN. Interpretation of the results will take into consideration other known oncogenic events occurring in these brain tumor cell lines. Moreover, it is recognized that our comparison of U87 cells +/- PTEN reconstitution will be straight-forward, the comparison of PTEN reconstitution between cell lines (U87, U373, U251, T98G) will be difficult. The puromycin selection of cells *in vitro* expressing mutants of PTEN or AKT may introduce some artifacts hence we will also study these PTEN mutants in an ecdysone-induced expression system as described in Preliminary Data, Figure 4E. This will allow for tumor growth *in vitro* or *in vivo* in the absence of PTEN or AKT mutant expression. At different time points after implantation we can induce PTEN or AKT expression by treating the cells or mice with ecdysone and examine outcome as relates to angiogenesis and tumor growth. We will also study these cell lines *in vitro* for the immediate effect of PTEN expression on biochemical parameters including phosphoAKT, TSP1 and p53 expression, etc. Lastly, we will generate in each case stable glioma cell lines transduced with empty retroviral vectors for comparison.

PTEN may control tumor growth, angiogenesis and survival of mice through the control of tumor cell proliferation and/or survival. So far our analysis of PTEN expressing tumor tissue argues strongly against this possibility. The tumor cells proliferate similarly *in vitro* (Figure 2A) and we see no different in apoptotic rates in cells *in vitro* (data not shown). To examine this possibility *in vivo* we have performed an analysis of cell cycle kinetics in tumors using *in vivo* BrdU labeling followed by anti-BrdU immunohistochemical staining and propidium iodide analysis of DNA content as well as immunohistochemical analysis of Ki67 and quantitation of mitotic index by H & E. Our data demonstrate a similar level of proliferation of tumor cells in PTEN deficient versus PTEN reconstituted tumors (data not shown). We will perform a comparison of apoptosis by the TUNEL method and DAPI staining as well as perform caspase 3 and DNA laddering analysis on tumor tissue. So far using the first two methods we see no significant difference in the frequency of apoptosis *in vivo* (data not shown). Hence so far we can conclude that the difference in tumor growth rate observed in Figure 3A-B is not due to differences in cell cycle entry or apoptotic frequency and seems to relate to angiogenesis (Figure 4A-C). Others laboratories have reported that PTEN exerts a control over both proliferation and apoptosis (19, 21, 71). It is possible that cell-type specific differences exist for different tumor types and that PTEN may exert an effect on cell growth in certain tumor cells via the control of angiogenesis. However we must admit that it is not completely clear why the PTEN reconstituted tumors grow so slowly *in vivo* (Figure 3). In Aim 2 we will address this issue by the forced expression of activated PI-3 kinase and AKT mutants in the PTEN reconstituted tumors. If PI-3 kinase and/or activated AKT can rescue the *in vivo* growth suppression in PTEN reconstituted U87 cells this would confirm that this signaling axis in involved. This will be particularly interesting if they are induced to grow based on increased angiogenesis and not via augmented proliferation as measured by *in vivo* BrdU staining.

Aim 2. We will investigate the role of PI-3 kinase and/or AKT kinase in control of tumor-induced angiogenesis, thrombospondin expression. We will correlate these data with regulation of p53/TSP1 expression and tumor growth and animal survival.

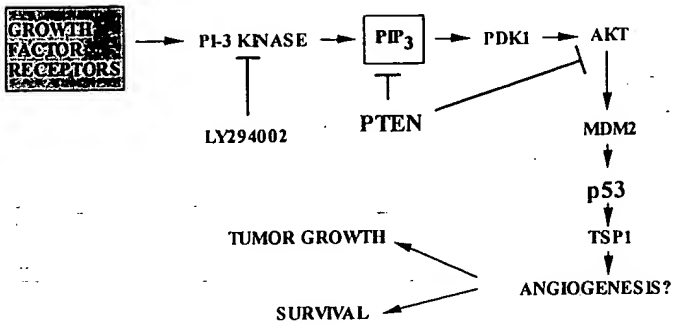

Figure 7. Postulated signaling pathways by which PTEN negatively controls PIP_3 and AKT activation leading to the suppression of angiogenesis. The data support a role for PTEN in the regulation of PIP_3 pathway and angiogenesis. In Aim 2 we will test this hypothesis using mutants of PTEN, AKT and PI-3 kinase. We will continue to explore a mechanistic link between PTEN, MDM2, p53, TSP1 and angiogenesis.

What is the role of AKT and/or PI-3 kinase in tumor induced angiogenesis? Based on our preliminary data, it appears that PTEN exerts control over angiogenesis through an effect upon PIP_3 dephosphorylation and to a lesser extent through its protein phosphatase activity (Figure 4A-C). This is based on our data that the G129E mutation in PTEN (a mutation which only affects PIP_3 phosphatase function) results in an angiogenic tumor (Figure 4C) comparable to the R130M mutant of PTEN which is catalytically dead. Other experiments performed by Myers et al, demonstrated the capacity of the G129E PTEN to dephosphorylate acidic peptide substrates *in vitro* (13). It is not clear from our data or from the literature which component of PI-3 kinase signaling, PIP_3 or AKT inactivation, is critical for the tumor suppressor function of PTEN *in vivo*. This is a critically important question to address. It is formally possible that the G129E mutant may be defective in the dephosphorylation of protein substrates *in vivo* including PDK-1 and AKT. This has not been formally tested *in vivo*. If so, the augmented phosphoAKT levels may reflect a dual defect and the phenotype may represent an effect of activated PIP_3 and activated AKT. To bolster this argument we must examine this question using activated and inhibitory mutants of PI-3 kinase and/or PDK1/AKT to prove this point.

Based on the preliminary data shown above in Figures 2-5, we anticipate that mutations in PTEN control angiogenesis as it relates to microvessel density through the deregulation of PIP_3 catalysis and not through loss of capacity to dephosphorylate acidic peptide sequences within proteins. This conclusion is based on the observation that the PTEN mutant, G129E, which has lost PIP_3 phosphatase function but retains protein phosphatase activity when expressed in U87MG cells does not suppress the angiogenic activity of tumors *in vivo* (Figure 4A-C). If the capacity to control the dephosphorylation of protein substrates was the central control point for PTEN action we would expect the G129E mutant would reduce the angiogenic response. As mentioned above, the G129E mutant may not display normal catalytic activity towards all protein substrates *in vivo*. Since Myers et al only looked at one substrate *in vitro* (13), it will be necessary to generate other evidence that PI-3 kinase and/or AKT control angiogenesis. Our model is an ideal system in which to test this hypothesis. We suggest that the PI-3 kinase will activate angiogenesis in our tumor model. To further evaluate this possibility we will express activating mutants of PI-3 kinase and AKT in U87 cells which express the wild type PTEN. In these cells, we will examine phospho-AKT, PDK1 activity and PIP_3 levels to determine if activated PI-3 kinase can induce the activation of PDK1 and AKT.

We will generate stable glioma cell lines (U87, U373, U251, T98G) expressing activated and dominant negative mutant of AKT or PI-3 kinase. The mutants of AKT will include a myristoylated version of AKT, termed, myr-AKT (Dr. David Donner, letter of collaboration) in which the PH domain of AKT is replaced by the SRC myristoylation signal subcloned into pBabe-neo. This mutant will constitutively localize to membrane as an activate AKT enzyme without interaction with PIP_3. The kinase dead AKT (K179M mutant will be subcloned into pBabe-neo plasmid. PI-3 kinase mutants used will be the deltap85 construct and p110.CAAX activated mutant (56). We will determine the effects of the deltap85, p110.CAAX, KD-AKT and myr-AKT expression on the extent of angiogenesis and TSP1 expression in U87 cells. For these experiments we would like to express the activated mutants of AKT and PI-3 kinase in glioma cell lines already expressing the wild type PTEN protein (Figure 2). We will ask whether the suppression of angiogenesis induced in these U87 cells can be broken by expression of an activated AKT or p110.CAAX protein. In contrast our strategy for evaluation of dominant negative mutants of AKT or PI-3 kinase will be to express these mutants in the parental glioma cell line where the angiogenic activity is high (Figure 4A-C) and determine if these mutants have the capacity to suppress angiogenesis, tumor growth and animal survival following intracranial implantation. We will correlate the observation made *in vivo* with biochemical analysis of mutant expressing glioma cells both in tissue culture and by analysis of tumor tissue. We will measure AKT activity by phosphoAKT blots and by *in vitro* kinase assay. We will assess the effects of AKT and PI-3 kinase mutant expression on TSP1 expression and p53 luciferase reporter activity and look at effects on nuclear MDM2/p53 levels. We will correlate angiogenesis and TSP1 with tumor growth and animal mortality in our nude mouse model (Figure 3-5).

Anticipated Results. These results will support or deny our view that glioma-induced angiogenesis is mediated through the deregulation of PI-3 kinase and/or AKT. These experiments will impact on Aim 3 where we propose to determine therapeutic activity of PI-3 kinase inhibitors against PTEN deficient tumors. If PTEN suppresses angiogenesis by inhibiting the PI-3 kinase axis, we would expect that PI-3 kinase inhibitors would inhibit angiogenesis. PI-3 kinase inhibitors would not affect the tyrosine phosphorylation state of proteins like focal adhesion kinase (FAK), SHC or SRC. Yamada has suggested that the tumor suppressive effects of PTEN may be exerted through its capacity to control protein tyrosine phosphorylation state of certain proteins (72). This may be true for certain phenotypes of tumor e.g. tumor cell motility and invasion and metastasis are likely controlled by these kinase cascades. The experiments defined in Aims 1 and 2 will help to determine whether the angiogenic phenotype of PTEN deficient tumors is mediated through PIP_3 phosphatase, AKT phosphatase or tyrosine phosphatase effects. Moreover , if the expression of dominant negative PI-3 kinase and/or dominant negative AKT results in the inhibition of the glioma induced angiogenic response this will provide "proof of concept" that the PI-3 kinase/AKT pathway is major control point for tumor-induced angiogenesis and strongly support experiments planned in Aim 3. If the expression of activated mutants of AKT or PI-3 kinase can interfere with PTENs capacity to suppress angiogenesis this would prove that PTEN suppresses angiogenesis via a control of PIP_3 pathways.

Alternatively, the effect of PTEN on angiogenesis may relate to the control of protein phosphorylation events other than AKT including evidence that PTEN can dephosphorylate tyrosine phosphorylated residues within protein tyrosine kinases like focal adhesion kinase (FAK) and Src kinase as well as adapter proteins, Shc (72). The capacity of PTEN to dephosphorylate FAK and SHC has not been demonstrated *in vivo*. It is also possible that other domains of PTEN, the tensin/auxilin homology domain may exert some activity on cytoskeleton separate from catalytic phosphatase domain as suggested by some of our data (Figure 3A) and Maier et al (73). These alternative activities must be considered if results from Aims 1 and 2 suggest effects other catalytic function of PI-3 kinase and AKT are responsible for stimulation of angiogenesis. To this end we will evaluate the phosphorylation state of FAK, SHC and SRC proteins and examine the SHC-GRB2 interaction in glioma cells. It is our contention that the effect of PTEN on tyrosine kinase pathways in more involved in tumor cell migration and invasion than transcriptional control of angiogenic factors (data not shown). Another possible downstream effector of PI-3 kinase which could contribute to the angiogenic response independent of AKT is the PDK1 kinase. Accordingly we will prepare reagents necessary to explore PDK1 (in

collaboration with Dr. David Donner) as the target for PTEN control as it relates to angiogenesis and TSP1 expression.

Since a catalytically activated mutant of AKT would act downstream of PIP_3 and hence downstream of PTEN the expression of this mutant should release the control exerted by PTEN over angiogenesis if angiogenesis is controlled by AKT. The activated mutant of AKT would not affect upstream events controlled by PIP_3 or PTEN. Therefore, if the control point for angiogenesis and TSP1 is upstream of AKT or on a separate pathway e.g. protein dephosphorylation of FAK or SHC then the activated mutant of AKT will not release the control exerted by wild type PTEN on angiogenesis (Figure 4). That is to say that if AKT activation is inducing the phosphorylation of MDM2 and the degradation of p53 which then leads to decreased TSP1, then the activated AKT mutant should decrease TSP1 expression in U87 cells and lead to increased angiogenic activity. If the control point for angiogenesis is not controlled by AKT and is controlled by PIP_3 levels unrelated to AKT activation, the expression of activated AKT in wild PTEN expressing cells should display a low angiogenic activity despite having low levels of TSP1 expression. This result would effectively dissect the effect of AKT activation on TSP1 expression from an effect on angiogenesis and lead to other targets for exploration. Again it is appreciated that PTEN may have numerous targets within the cell any of which could exert control over the angiogenic phenotype. Since we envision the PTEN effect to be an intracellular effect within the tumor cell, it is our view that a major effect of PTEN mutation is to alter the expression and secretion of angiogenic proteins which then mediate multiple effects upon the stromal compartment of the tumor i.e. endothelial cells, macrophages, etc. It is appreciated that other signaling pathways e.g. cytoskeletal reorganization, cell migration and matrix degradation may be controlled by PTEN. These may contribute to regulation of TSP1 and angiogenesis.

Potential problems and pitfalls. It is possible that PTEN exerts significant effects through activities unrelated to its protein and/or lipid phosphatase catalysis. This is suggested by the effect of catalytically dead PTEN (G129E,R130M) on growth of the U87 tumors *in vivo* (Figure 3A-B). Others have suggested that PTEN may exert some negative effects on cell invasion through a noncatalytic mechanism (73). This is a difficult idea to test. In certain assays we observe that the catalytically dead mutant (R130M) affects the capacity of glioma cells to migrate (integrin) whereas the loss of PIP_3 phosphatase alone does not. Hence it is apparent that for certain phenotypes the capacity to dephosphorylate proteins is the critical control point for PTEN. It follows that other regions of PTEN may also regulate cell function. It is reasoned that these effects of catalytically dead PTEN (R130M and G129E) may occur via occupancy of membrane docking sites on plasma membrane and that PTEN may serve a key scaffolding function. It is also possible that PTEN dephosphorylation events subserve a positive role in cell signaling. This could relate to phosphorylation/dephosphorylation cycles which may impart a dynamic element to PTEN signaling as suggested in Figure 8. If we do away with capacity of PTEN to attach to the membrane, we predict that PTEN would not exert an effect on PIP_3. This idea is supported in the literature by the observation that the PDZ deletion despite having catalytic activity will not suppress membrane ruffling which suggests a defect in regulation of RAC (62). Hence we propose to generate a mutant of PTEN where the G129E and R130M mutants are engineered without a PDZ domain and determine the effect of these mutants on tumor growth *in vivo* as described in Figure 3A. As mentioned before, all of C terminal mutants and point mutants must be studied for capacity to associate with membrane. The expected result based on our data in Figure 3A is that the growth inhibitory effect will not be present in the R130M-delta-PDZ mutant. We expect this mutant to have similar high angiogenic activity as compared to the R130M mutant (Figure 4C).

Aim 3. Determine if PI-3 kinase inhibitors have effects on angiogenesis and glioma growth *in vitro* or *in vivo* and whether and how these inhibitors affect mortality *in vivo*. Test PI-3 kinase inhibitors (LY294002) for potential therapeutic activity in our orthotopic brain tumor model. We will evaluate adult and pediatric glioma tumor tissue for mutations in PTEN by RT-PCR, SSCP and immunohistochemical staining and examine these tumors for evidence of altered PI-3 kinase/AKT signaling.

Aim 3. We will determine the effects of PI-3 kinase inhibitors on angiogenesis.

Experiments. We will study the effects of LY294002 (2-(4-morpholinyl)8-phenyl-4H-1-benzopyran-4-one), *in vitro* on U87, U373 and U251 cells reconstituted with PTEN, activated mutants of PI-3 kinase or AKT or dominant negative mutants of PI-3 kinase or AKT. We will examine the effects of LY294002 on the T98G glioma cell line which is p53 deficient. We will examine in these different cell lines sensitivity to LY294002 (dose response curve) as it relates to effects of LY294002 on TSP1 expression and p53 transcription. The range of doses used *in vitro* will be from 10-50 uM doses which are known to inhibit the growth factor induced activation of AKT in several systems (66). We will measure the effects of LY294002 on PI-3 kinase activity and phosphoAKT and correlate these results with effects on p53 transcription, TSP1 expression and MDM2 localization.

Only one previous study has examined the effect of LY294002 *in vivo* (74). To examine the effect of LY294002 in our animal model, mice implanted with U87 or other glial tumor cells reconstituted with empty vector or PTEN mutants will treated with the PI-3 kinase inhibitor, LY294002. The dose of LY294002 used will be used based on *in vitro* and one published report of its use in nude mice (74). We will begin treatment with LY294002 two days after tumor implantation and continue for 2 weeks duration. We will perform a dose-finding experiment where we begin with 50 mg/kg and escalate the dose 15-20% per treatment cycle in an effort to determine if toxicity is observed for this agent in nude mice. Hu et al recently reported one of the first *in vivo* experiments with the PI-3 kinase inhibitor, LY294002, treatment of nude mice with ovarian tumor cells resulted in decreased ascites and improved survival (74). These investigators used 200 mg/kg body weight (1 mg dose given twice a day for 3 weeks) with no untoward effects noted. In our experiments we will begin at 50 mg/kg and escalate to 200 mg/kg. We will measure in the subcutaneous tumor tissues the levels of phosphoAKT as described above (Figure 3C). We will examine effects of LY294002 on microvessel density, TSP1 expression, tumor growth and mortality of mice implanted intracranially with U87, U373, U251 and T98G tumors. We will determine phosphoAKT levels in tumor tissue obtained from subcutaneous tumors and brain tumors. Since we are unaware of the capacity of LY294002 to cross the blood brain barrier we will examine both skin and brain tumor tissues simultaneously.

An important component of the orthotopic brain tumor analysis will be our capacity to examine tumor cells which have suppressed levels of PIP_3 versus phosphoAKT (Figure 3C) for sensitivity to these inhibitors and correlate effects downstream e.g. angiogenesis, TSP1 expression, tumor growth and animal mortality. These results will be important for several reasons: 1) Sensitivity to LY294002 and other analogs will in cells with different PTEN profiles will to some extent validate the specificity of these inhibitors for PI-3 kinase suppression 2) PI-3 kinase inhibitor effects on TSP1 and p53 transcription should be related to relative suppression of PIP_3 levels this will serve to confirm observations made in Aim 1 and 2, where we will also correlate the effects of different PTEN/PI-3 kinase/AKT mutants with PIP_3/AKT activation, TSP1 expression, angiogenesis, tumor growth and animal mortality.

Anticipated Results. The interpretation of these results should be straight-forward and validated by our observations in Aims 1 and 2 using PTEN mutant and AKT and PI-3 kinase mutants. We predict that PI-3 kinase inhibitors will block the angiogenic response of gliomas in our *in vivo* model and inhibit tumor growth. Based on our preliminary data, we predict that like the phenotype of the G129E PTEN mutant angiogenesis is predominantly controlled by PIP_3 phosphatase activity of PTEN. Likewise since we hypothesize that TSP1 is major negative regulator of angiogenesis that LY294002 will increase TSP1 expression. Accordingly, LY294002 will result in the induction of p53 levels and elevate MDM2 levels via the induced phosphorylation of MDM2 and translocation of MDM2 into the nucleus. We predict that treatment of U87 cells with LY294002 will induce the p53 luciferase reporter and augment TSP1 secretion and expression again strengthening our idea that AKT and PTEN are in control of p53 functional activity and TSP1 within the cell (Figure 7). It is also likely that LY294002 will inhibit the synthesis and secretion of VEGF and bFGF. This we expect will correlate with the inhibition of angiogenesis as measured by microvessel quantitation (Figure 4A-C). If so, this would provide the preclinical data to launch a phase I clinical trial for PI-3 kinase inhibitors for the treatment of malignant gliomas. Finally as relates to the T98G cell line, if this tumor is highly angiogenic in our animal model and if the control of angiogenesis is

dependent upon the p53 induction of TSP1 then we predict that treatment of this cell with LY294002 will not induce TSP1 or suppress angiogenesis. Alternatively if some other mechanism exists for PTENs control over TSP1 and/or angiogenesis, other than p53, then it is possible that LY294002 may suppress the T98G glioma-induced angiogenesis and growth. To confirm this idea, we will express the wild type p53 in T98G cells expressing wild type PTEN to determine if p53 will reconstitute the signal to TSP1. This will constitute "proof of concept' that the PTEN-p53-TSP1 pathway is indeed operative.

Potential problems and pitfalls. Inhibitors are pleiotrophic agents and the PI-3 kinase inhibitor, LY294002 can block other kinases including the DNA-activated protein kinase (DNA-PK). The IC_{50} of this inhibitor is 1.4 uM and at 50 uM it does not inhibit the EGF receptor kinase or SRC nor does it affect PKC, PI-4 kinase, ribosomal S6 kinase or MAP kinases (75, 76). The IC_{50} for LY294002 is significantly lower than the IC_{50} for wortmannin. We will compare and contrast the effects of LY294002 *in vivo* with effects of wild type PTEN reconstitution (Aim 1) and expression of activated mutants of PI-3 kinase and AKT (Aim 2) on tumor induced angiogenesis. We will correlate the effect of LY294002 with phospho-AKT evaluation in an effort to internally validate our observations with inhibitor. For example we would perform a dose response curve of U87 glioma cells sensitivity to LY294002 *in vivo* as relates to tumor growth and angiogenesis. We will examine the effect of LY294002 for capacity to inhibit PI-3 kinase and AKT as compared to EGF receptor, PDGF receptor or SRC *in vivo* and *in vitro* by performing in vitro kinase assays. We would predict that cells reconstituted with wild type PTEN and not catalytic mutants of PTEN would be more sensitive to the LY294002 agent as it relates to tumor growth, angiogenesis, TSP1 expression pattern. In this way Aims 1-3 are highly inter-related, results from Aim 1 and 2 will be validated using PI-3 kinase inhibitors and vice versa.

Analysis of human glial tumors (Clinical samples) (Dr. B. Azzarelli)

Our planned evaluation of human tumor tissue for PTEN mutations and PI-3 kinase AKT activation has two goals: 1) to compare the results from our orthotopic brain tumor model with pattern of PTEN expression and PI-3 kinase/AKT activity in primary human tumor tissue 2) To examine relationships between PTEN, phosphoAKT, p53 and TSP1 in human tumors and angiogenesis as prelude to clinical application of PI-3 kinase inhibitors. In these studies, we will compare clinical grade I-IV glioma tumor tissue (WHO classification system for gliomas)(Dr. Biaggio Azzarelli, IU School of Medicine, Indianapolis, IN) (letter of collaboration). RT-PCR will be employed using PCR primers to amplify the full length PTEN cDNA which will allow us to screen a large panel of paraffin embedded archival tumors for presence or absence of full length PTEN message. This will allow us to detect truncated PTEN as result of frame-shift mutations in PTEN (50% incidence of nonsense mutation in PTEN mostly within the C-terminus) (25). We have established through a strong collaboration with Dr. Biaggio Azzarelli, Neuropathology, Indiana University School of Medicine (see letter of collaboration) a system by which we will receive fresh frozen tumor tissue from every glial tumor removed at time of surgery. These tissues will be processed for frozen section immunohistochemistry (as defined below) and a sample will be processed to extract DNA, RNA and protein for analysis for further investigation into PTEN and AKT status in glioma patients. The immunohistochemical staining of frozen OCT blocks of tumor will allow us to evaluate the morphology of tumor and correlate with routine staining for H&E, Ki67, CD31, factor VIII, GFAP and mitotic indices and compare with more specialized immunohistochemical stains which directly relate to the above specific aims 1-2 of this proposal i.e. PTEN, p53, MDM2, TSP1 and EGF receptor. Tissue specimens will be taken at time of surgery for frozen block and paraffin embedding for further analysis. Fresh tissue lysates will be prepared for phospho-AKT and TSP1 immunoblot analysis as described in Figure 2C above. Importantly, these analyses will be compared to a parallel analysis of tissues obtained from the orthotopic implanted brain tumors +/- PTEN (Figure 4). Point mutations, which account for 50% of PTEN mutations so far detected (25) will not be detected in our antibody or RT-PCR screens and perhaps be the most interesting from structure/function standpoint given current knowledge of the crystal structure of PTEN (25). We will screen DNA from these tumors by SSCP as described for evidence of point mutations in PTEN. DNA samples will then be subjected to direct sequence analysis of exons 2-9 to confirm possible specific site of

mutation (part of a separate proposal). This is necessary since it is known that in humans a transcribed pseudogene exists.

Anticipated results. Our prediction would be as suggested by the work of Raffel et al (1, 2) and as suggested by data from our animal model that a loss of PTEN would be associated with more aggressive more angiogenic glial tumors. Previous studies cited above did not evaluate the angiogenic profile or AKT status of human tumors. We would expect that PTEN mutation would be associated with high levels of phospho-AKT as suggested by Holland et al in another animal model. Low grade gliomas which in previous reports do not have PTEN mutations (1, 2) would be expected to have low microvessel density and + PTEN staining. In preliminary experiments from our animal model we observe that loss of PTEN is associated with dramatic increase in Ki67 stain as was observed in the PTEN heterozygous knockout mice (24). Interestingly, despite great differences in Ki67 staining the mitotic index was similar in PTEN positive and null tumors. If PTEN deficient tumors are discovered we will send these tumors to Dr. C. David James, Mayo Clinic Research Foundation, Rochester, MN for DNA sequence analysis of exons 2-8, to determine mutations (not part of this proposal). We will examine levels of TSP1 expression to correlate this result with our animal model. Lastly, the targeted phenotypic analysis of malignant brain tumors may allow for some level of prediction of sensitivity for the use of PI-3 kinase inhibitors or PTEN gene therapy for the future. This analysis will be carried along as we move forward to develop PI-3 kinase inhibitors for the treatment of malignant gliomas in man. It is possible that patients with different PTEN mutations and AKT phenotypes, like the results predicted in our animal model, may show differential sensitivity and clinical responsiveness to PI-3 kinase inhibition. We will examine carefully each PTEN mutation noted in our glial tumor samples in the context of the known crystal structure of PTEN (25). The most interesting and informative mutations will be constructed and expressed in PTEN null glioma cell lines for analysis *in vitro* and in our orthotopic animal model.

Summary and projected impact of the work: The PTEN gene is mutated at increasing frequency in a number of types of human malignant disease including brain, prostate, breast, endometrial, hepatic, colon, leukemias and lymphomas and bladder cancer. Hence the elucidation of how different mutations in PTEN affect different components of tumor progression will provide useful prognostic and therapeutic information for the clinical management of these diseases. Certain PTEN mutations may define augmented angiogenic properties (e.g. G129E, R130M) of tumors and define specific anti-angiogenic interventions; others may define invasion, migration and/or metastatic or control the growth potential of tumors (regions outside of catalytic domain)(C2, PDZ, C terminal tail domains). Along with other oncogenic signals: EGF receptor, RAS, PI-3 kinase, AKT, p53, p16, MDM2, MYC; PTEN may contribute to tumor progression in qualitatively different ways in different tumors depending the complement of cooperating oncogenes and mutations in suppressor oncoproteins. Importantly defining the quality of these interactions will provide the substratum on which we build a tailored approach to the therapeutic management of malignant brain tumors and other malignant diseases. Finally mutations in PTEN deregulate PIP_3 pathways within the cell. Finally, we suggest that the entry of PI-3 kinase inhibitors into therapeutic oncology may offer exciting prospects for therapeutic attack of tumors deficient in PTEN. It also opens the prospect for chemoprevention using long term use of PI-3 kinase inhibitors in select patients to prevent progression to a non-treatable angiogenic/metastatic state.

Methods.

a. **Constructs, cell lines and reagents.** Wild type PTEN or mutant PTEN (G129E, R130M, C124S) cDNAs was subcloned into the pBabe-puro retroviral expression vector. Stable clones of U87MG cells were established under puromycin selection (2 ug/ml). We also will use a retrovirus based ecdysone-induced expression system to express mutants of PTEN, AKT and PI-3 kinase as described in Figure 4D above). Antibodies against PTEN, AKT and phospho-AKT (New England Biolabs, #9270), TSP-1, (Calbiochem, #605230), VEGF, (Santa Cruz, SC-507), bFGF (Santa Cruz, SC-79), HIF1α, (Novus Biologicals, NB 100-105). Mutagenesis will be performed using the Quick-Change mutagenesis kit, (Stratagene) according to manufacturer. Each mutant will be sequenced following PCR subcloning into the TA cloning vector prior to subcloning into mammalian expression vector

pBABE. For AKT and PI-3 kinase mutants we will subclone these into the pBABE-zeo and pBABEneo for selection under zeocin and/or neomycin for double transfection into PTEN reconstituted-puromycin resistant cells. The glioma cell lines used will be U87MG, U373MG, and U251MG obtained from the ATCC (all null for both PTEN alleles, wild type p53 expression). The T98G cell line will come from ATCC (null for PTEN, null for p53).

b. **Tumor implantation.** U87, U373, U251 or T98G cells will be cultured in fresh medium for 24 hours and harvested, adjusting the cell concentration to 1×10^6 in 10 ul of RPMI medium. Mice, under general anesthesia were placed into the stereotactic device (model 963, Kopf, Tugunga, CA). Stereotactically controlled drill assembly was used to provide a hole 0.3 mm deep and of 0.8 mm diameter in cranium at a position 0.5 mm anterior and 1.2 mm lateral to the bregmal anatomical landmark. Tumor cells (1×10^6) were introduced slowly through a 10 ul Hamilton syringe at a depth of 2.5 mm at a rate of 2 ul per minute. We then slowly removed the needle at a rate of 0.5 mm/min. After needle removal we seal the hole with bone wax and close the incision with a wound clip. In most of the mice, 5×10^6 tumor cells were implanted subcutaneously into the right flank to monitor tumor volume and to perform biochemical and immunohistochemical analysis of tumor tissue.

c. **Biochemical analysis.** Immunoblots were performed on cell lysates obtained from U87 cells grown in tissue culture or from multiple cryostat sections of U87MG subcutaneous tumor tissues. A Bradford assay was performed to determine protein concentration of each lysate. Equivalent amounts of protein were resolved by SDS PAGE and transferred to nitrocellulose. Membranes were probed with antisera specific for PTEN, AKT, phospho-AKT or TSP-1. The RNAase protection assay (RPA) was performed using a RPA III kit from (Ambion) according to the manufacture specifications. Briefly, 20 ug of total RNA was precipitated and resuspended in 10 ul of hybridization buffer containing the radioactive probe. The RNA is then heated to 95 degrees C for 10 min then hybridized for 16 hours at 42 degrees C. 150 ul of this mixture is treated with 1:100 dilution of RNAase in RNAase buffer for 30 minutes. RNAase is inactivated, RNA is reprecipitated and resolved on 5% acrylamide gel. RNA probes were synthesized using MAXI Script using PCR templates and T7 polymerase. The GAPDH probe was provided in the kit and TSP-1 probe represents a 590 nucleotide sequence in the 3' UTR of TSP-1 sequence. All probes were sequenced.

d. **Kinase and phosphatase assays.** We will use exogenous substrates in an *in vitro* kinase assay to quantitate AKT, PDK1 and PI-3 catalytic activity as described previously (77). Phosphatase assays will be carried out as described (64) using acidic peptide $polyglu_4tyr_1$ and PIP_3. To assess levels of GTP-RAC we will use PAK-PBD pull-down assay (Upstate #18943).

e. **Inhibitor experiments.** We will employ the PI-3 kinase inhibitor, LY294002 (Calbiochem) between 10-40 uM for *in vitro* experiments and 50-200 mg/kg body weight for animal studies.

f. **Immunohistochemical analysis.** Microvessel density (MVD) was determined for each subcutaneous tumor by CD31 staining, performed on cryostat sections (7 um), fixed in acetone, blocked in 1% goat serum and stained with anti-CD31 antibody (Pharmingen, #01951D). Antibody staining was visualized with peroxidase-conjugated anti-mouse and counter stained with hematoxylin. A negative control was performed on each tumor tissue stained with mouse IgG. Two sections from each tumor were scanned under low power magnification (x40) to identify areas of highest CD31 positive vessel density, followed by digitization of 3 fields from this area. The digitized images representing one 100x field were counted for number of CD31 positive vascular elements. Data was collected from two independent observers without knowledge of which tumors were viewed. The average number of microvessels per digitized 100x field will be determined for 5 tumors per experimental group and analyzed by Student's t-test. Neuropathologic evaluation of human brain tumor tissue will be carried out under the direction of Dr B. Azzarelli (see letter of collaboration). Briefly, frozen or paraffin embedded tissues will be processed for routine histopathology and RNA will be extracted for RT-PCR analysis using primers specific for PTEN sequence. The immunohistochemical analysis of Ki67, p53 and GFAP have been established in the neuropathology

laboratory under the direction of Dr B. Azzarelli and are functioning well at this time. The PCR conditions have been worked out using the U87 cell lines expressing PTEN or null for PTEN.

g. Statistical analysis of in vitro and animal data will be by the Student's t-test. Survival will be evaluated using Kaplan-Meier analysis.

Time frame for proposed studies:

Year 1. Generate epitope tagged wild type and site directed mutants of PTEN. Prepare activated and dominant negative mutants constructs of AKT and PI-3 kinase for expression in glioma cell lines. Begin collection of human brain tumor tissues. Express PTEN mutants in glioma cell lines.

Year 2. Continue to express activated mutants of PTEN, PI-3 kinase and AKT in clones of U87, U373, U251 and T98G cells. Determine effects of these mutants in PTEN on angiogenesis. Express TSP1 in glial cell lines and determine effect on tumor growth and angiogenesis. Effect of PTEN on p53 transcriptional activity and TSP expression in U87 compared with T98G cells. Continue analysis of human brain tumor tissues.

Year 3. Continue to analyze effect of PI-3 kinase inhibitors on tumor growth and angiogenesis, TSP1 and p53 expression in glioma cells and in the orthotopic brain tumor model. Continued analysis of PTEN and AKT mutants on glioma induced angiogenesis. Continue analysis of human brain tumor tissues.

Year 4.. Determine PTENs effect on TSP1 expression is via the effect on p53 transcription. Does PTEN control the TSP1 promoter. Affect of PTEN proves a role for PIP3 phosphatase activity in control of angiogenesis, tumor growth and survival. Continue analysis of human brain tumor tissues.

Year 5. Complete our evaluation of effects of mutant of AKT, PTEN and PI-3 kinase on angiogenesis. Complete analysis of PTEN mutations in adult and pediatric brain tumor tissue. Model analysis of PTEN mutants and incidence of PTEN mutations in human glial tumors.

Gender and Minority Inclusion. No Human Subjects are included in this research proposal.

Human Subjects. No human subjects will be involved in this research protocol.

Vertebrate Animals. (IACUC #2193) The proposed experiments will use nude/nude Balb/c mice and a stereotactic orthotopic brain tumor model. Although a significant amount of the project can be accomplished using tissue culture, our orthotopic animal model is the only method to evaluate the behavior of glioma tumor cells *in vivo*. There is no alternative to using animals to test our hypothesis. All procedures are performed with mice under anesthesia as defined by our Animal Care and Use Committee (IACUC regulations are strictly followed). Anesthesia will occur using nebutal (50/mg/kg/dose) titrated to achieve total anesthesia. Animals will be checked daily by myself or by qualified personnel in my laboratory. All animals are cared for in our laboratory animal resource center by trained technicians and supervised by two full-time veterinarians. The method of euthanasia used is CO_2 euthanasia followed by confirmation by cervical dislocation. This method has been approved by our Animal Care and Use Committee and is consistent with the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association.

Consultants/Collaborators (letters following literature cited)

Biagio Azzarelli, MD	Indiana University School of Medicine
Nicholas K. Tonks, PhD	Cold Spring Harbor Laboratories

David B. Donner PhD Indiana University School of Medicine

David A. Williams, MD Indiana University School of Medicine

Consortium/Contractual Agreements. Not applicable

Literature Cited.

1. Raffel, C., Frederick, L., O'Fallon, J. R., Atherton-Skaff, P., Perry, A., Jenkins, R. B., and James, C. D. Analysis of oncogene and tumor suppressor gene alterations in pediatric malignant astrocytomas reveals reduced survival for patients with PTEN mutations, Clin Cancer Res. *5:* 4085-90, 1999.
2. Rasheed, B. K., Stenzel, T. T., McLendon, R. E., Parsons, R., Friedman, A. H., Friedman, H. S., Bigner, D. D., and Bigner, S. H. PTEN gene mutations are seen in high-grade but not in low-grade gliomas, Cancer Res. *57:* 4187-90, 1997.
3. Frederick, L., Wang, X. Y., Eley, G., and James, C. D. Diversity and frequency of epidermal growth factor receptor mutations in human glioblastomas, Cancer Res. *60:* 1383-7, 2000.
4. Westphal, M. and Meissner, H. Establishing human glioma-derived cell lines, Methods Cell Biol. *57:* 147-65, 1998.
5. O'Rourke, D. M., Qian, X., Zhang, H. T., Davis, J. G., Nute, E., Meinkoth, J., and Greene, M. I. Trans receptor inhibition of human glioblastoma cells by erbB family ectodomains, Proc Natl Acad Sci U S A. *94:* 3250-5, 1997.
6. Rameh, L. E. and Cantley, L. C. The role of phosphoinositide 3-kinase lipid products in cell function, J Biol Chem. *274:* 8347-50, 1999.
7. Rameh, L. E., Rhee, S. G., Spokes, K., Kazlauskas, A., Cantley, L. C., and Cantley, L. G. Phosphoinositide 3-kinase regulates phospholipase Cgamma-mediated calcium signaling, J Biol Chem. *273:* 23750-7, 1998.
8. Hunter, T. Protein kinases and phosphatases: the yin and yang of protein phosphorylation and signaling, Cell. *80:* 225-36, 1995.
9. Li, J., Yen, C., Liaw, D., Podsypanina, K., Bose, S., Wang, S. I., Puc, J., Miliaresis, C., Rodgers, L., McCombie, R., Bigner, S. H., Giovanella, B. C., Ittmann, M., Tycko, B., Hibshoosh, H., Wigler, M. H., and Parsons, R. PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer [see comments], Science. *275:* 1943-7, 1997.
10. Steck, P. A., Pershouse, M. A., Jasser, S. A., Yung, W. K., Lin, H., Ligon, A. H., Langford, L. A., Baumgard, M. L., Hattier, T., Davis, T., Frye, C., Hu, R., Swedlund, B., Teng, D. H., and Tavtigian, S. V. Identification of a candidate tumour suppressor gene, MMAC1, at chromosome 10q23.3 that is mutated in multiple advanced cancers, Nat Genet. *15:* 356-62, 1997.
11. Li, D. M. and Sun, H. TEP1, encoded by a candidate tumor suppressor locus, is a novel protein tyrosine phosphatase regulated by transforming growth factor beta, Cancer Res. *57:* 2124-9, 1997.
12. Myers, M. P., Stolarov, J. P., Eng, C., Li, J., Wang, S. I., Wigler, M. H., Parsons, R., and Tonks, N. K. P-TEN, the tumor suppressor from human chromosome 10q23, is a dual- specificity phosphatase, Proc Natl Acad Sci U S A. *94:* 9052-7, 1997.
13. Myers, M. P., Pass, I., Batty, I. H., Van der Kaay, J., Stolarov, J. P., Hemmings, B. A., Wigler, M. H., Downes, C. P., and Tonks, N. K. The lipid phosphatase activity of PTEN is critical for its tumor supressor function, Proc Natl Acad Sci U S A. *95:* 13513-8, 1998.
14. Maehama, T. and Dixon, J. E. The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate, J Biol Chem. *273:* 13375-8, 1998.
15. Stambolic, V., Suzuki, A., de la Pompa, J. L., Brothers, G. M., Mirtsos, C., Sasaki, T., Ruland, J., Penninger, J. M., Siderovski, D. P., and Mak, T. W. Negative regulation of PKB/Akt-dependent cell survival by the tumor suppressor PTEN, Cell. *95:* 29-39, 1998.
16. Wu, X., Senechal, K., Neshat, M. S., Whang, Y. E., and Sawyers, C. L. The PTEN/MMAC1 tumor suppressor phosphatase functions as a negative regulator of the phosphoinositide 3-kinase/Akt pathway, Proc Natl Acad Sci U S A. *95:* 15587-91, 1998.

these lines are wild type for p53 and are null for PTEN. These data suggest that the U87MG cell line is deregulated by activated growth factor signals, resulting in the activation of PI-3 kinase/AKT pathways which are unopposed by mutations in PTEN (5)(Figure 1). It should be stated at the onset that the PI-3 kinase/PDK1/AKT pathways are highly complex and interrelated and most of the connections between them are poorly understood (6, 7).

PTEN is a tumor suppressor for glial tumors.

The reversible phosphorylation of proteins and lipids is critical to the control of signal transduction in mammalian cells and is regulated by kinases and phosphatases (8). The product of the tumor suppressor gene PTEN/MMAC (hereafter termed PTEN) was identified as a dual specificity phosphatase and has been shown to dephosphorylate inositol phospholipids *in vivo* (9-16). The PTEN gene, which is located on the short arm of chromosome 10 (10q23), is mutated in 40-50% of high grade gliomas as well as prostate, endometrial, breast, lung and other tumor types (9, 10, 17). In addition, PTEN is mutated in several rare autosomal dominant cancer predisposition syndromes, including Cowden disease, Lhermitte-Duclos disease and Bannayan-Zonana syndrome (18-21). Furthermore, the phenotype of PTEN-knockout mice revealed a requirement for this phosphatase in normal development and confirmed its role as a tumor suppressor (22-24).

PTEN is a 55kDa protein comprising an N-terminal catalytic domain, identified as a segment with homology to the cytoskeletal protein tensin and containing the sequence $HC(X)_5R$, which is the signature motif of members of the protein tyrosine phosphatase family, and a C-terminal C2 domain with lipid-binding and membrane-targeting functions (25). A five amino acid sequence in the extreme C-terminus of PTEN encodes a PDZ domain-binding site (Figure 1). Recent evidence points to a role for both the C2 and PDZ domains for membrane localization and protein stability (26). A recent report describes 3 phosphorylation sites within the C-terminus of PTEN which regulate protein stability suggesting a potential mechanism for regulation (27). PTEN is a dual specificity phosphatases that displays a pronounced preference for acidic substrates (12). Importantly, PTEN possesses lipid phosphatase activity, preferentially dephosphorylating phosphoinositides at the D3 position of the inositol ring. It is the only enzyme known to dephosphorylate the D3 position in inositol phospholipids, suggesting that PTEN may function as a direct antagonist of PI-3 kinase and PIP_3-dependent signaling events (14, 20). Reconstitution of PTEN in tumor cells that carry mutations in the PTEN gene, have established that this phosphatase regulates the PI-3 kinase-dependent activation of AKT, a major player in cell survival (13, 21). Mutations in the C-terminal C2 domain of PTEN occur in human tumors and have been observed to abrogate the growth suppressive effects of PTEN *in vivo* (25). Despite progress in understanding the biochemistry of PTEN, the role of this phosphatase in tumor progression, particularly as it relates to angiogenesis, cell growth and/or animal survival, remains unclear.

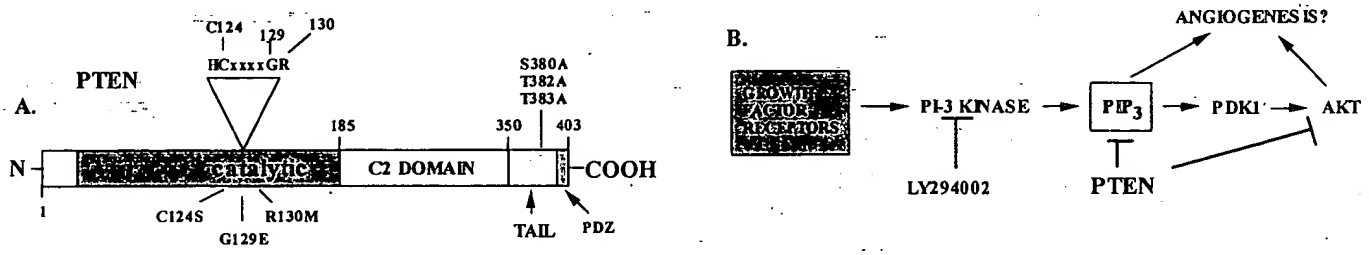

Figure 1. Schematic representation of PTEN domain structure and signaling. A. We show the domain structure of PTEN showing position of N-terminal Tensin/Catalytic domains with consensus phosphatase sequence containing mutations (G129E, R130M, C124S). In the C-terminus we diagram the C2 and PDZ domains with phosphorylation sites and positions of PTEN mutants cited in this application. B. Linear diagram showing known connections between PTEN and PI-3 kinase cascade including the downstream effector, AKT. We show hypothetical sites of action of PTEN and LY294002 on growth factor signaling pathways which may impact on angiogenesis.

17. Maier, D., Zhang, Z., Taylor, E., Hamou, M. F., Gratzl, O., Van Meir, E. G., Scott, R. J., and Merlo, A. Somatic deletion mapping on chromosome 10 and sequence analysis of PTEN/MMAC1 point to the 10q25-26 region as the primary target in low- grade and high-grade gliomas, Oncogene. *16:* 3331-5, 1998.
18. Liaw, D., Marsh, D. J., Li, J., Dahia, P. L., Wang, S. I., Zheng, Z., Bose, S., Call, K. M., Tsou, H. C., Peacocke, M., Eng, C., and Parsons, R. Germline mutations of the PTEN gene in Cowden disease, an inherited breast and thyroid cancer syndrome, Nat Genet. *16:* 64-7, 1997.
19. Myers, M. P. and Tonks, N. K. PTEN: sometimes taking it off can be better than putting it on [editorial], Am J Hum Genet. *61:* 1234-8, 1997.
20. Maehama, T. and Dixon, J. E. PTEN: a tumour suppressor that functions as a phospholipid phosphatase, Trends Cell Biol. *9:* 125-8, 1999.
21. Cantley, L. C. and Neel, B. G. New insights into tumor suppression: PTEN suppresses tumor formation by restraining the phosphoinositide 3-kinase/AKT pathway, Proc Natl Acad Sci U S A. *96:* 4240-5, 1999.
22. Podsypanina, K., Ellenson, L. H., Nemes, A., Gu, J., Tamura, M., Yamada, K. M., Cordon-Cardo, C., Catoretti, G., Fisher, P. E., and Parsons, R. Mutation of Pten/Mmac1 in mice causes neoplasia in multiple organ systems, Proc Natl Acad Sci U S A. *96:* 1563-8, 1999.
23. Suzuki, A., de la Pompa, J. L., Stambolic, V., Elia, A. J., Sasaki, T., del Barco Barrantes, I., Ho, A., Wakeham, A., Itie, A., Khoo, W., Fukumoto, M., and Mak, T. W. High cancer susceptibility and embryonic lethality associated with mutation of the PTEN tumor suppressor gene in mice, Curr Biol. *8:* 1169-78, 1998.
24. Di Cristofano, A., Pesce, B., Cordon-Cardo, C., and Pandolfi, P. P. Pten is essential for embryonic development and tumour suppression, Nat Genet. *19:* 348-55, 1998.
25. Lee, J. O., Yang, H., Georgescu, M. M., Di Cristofano, A., Maehama, T., Shi, Y., Dixon, J. E., Pandolfi, P., and Pavletich, N. P. Crystal structure of the PTEN tumor suppressor: implications for its phosphoinositide phosphatase activity and membrane association, Cell. *99:* 323-34, 1999.
26. Wu, X., Hepner, K., Castelino-Prabhu, S., Do, D., Kaye, M. B., Yuan, X. J., Wood, J., Ross, C., Sawyers, C. L., and Whang, Y. E. Evidence for regulation of the PTEN tumor suppressor by a membrane-localized multi-PDZ domain containing scaffold protein MAGI-2, Proc Natl Acad Sci U S A. *97:* 4233-8, 2000.
27. Vazquez, F., Ramaswamy, S., Nakamura, N., and Sellers, W. R. Phosphorylation of the PTEN Tail Regulates Protein Stability and Function, Mol Cell Biol. *20:* 5010-5018, 2000.
28. Folkman, J. Tumor angiogenesis: therapeutic implications, N Engl J Med. *285:* 1182-6, 1971.
29. Brem, S., Cotran, R., and Folkman, J. Tumor angiogenesis: a quantitative method for histologic grading, J Natl Cancer Inst. *48:* 347-56, 1972.
30. Hanahan, D. and Folkman, J. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis, Cell. *86:* 353-64, 1996.
31. Brooks, P. C., Montgomery, A. M., Rosenfeld, M., Reisfeld, R. A., Hu, T., Klier, G., and Cheresh, D. A. Integrin alpha v beta 3 antagonists promote tumor regression by inducing apoptosis of angiogenic blood vessels, Cell. *79:* 1157-64, 1994.
32. Plate, K. H., Breier, G., Weich, H. A., and Risau, W. Vascular endothelial growth factor is a potential tumour angiogenesis factor in human gliomas in vivo, Nature. *359:* 845-8, 1992.
33. Jiang, B. H., Zheng, J. Z., Aoki, M., and Vogt, P. K. Phosphatidylinositol 3-kinase signaling mediates angiogenesis and expression of vascular endothelial growth factor in endothelial cells, Proc Natl Acad Sci U S A. *97:* 1749-53, 2000.
34. Giri, D. and Ittmann, M. Inactivation of the PTEN tumor suppressor gene is associated with increased angiogenesis in clinically localized prostate carcinoma, Hum Pathol. *30:* 419-24, 1999.
35. Furnari, F. B., Lin, H., Huang, H. S., and Cavenee, W. K. Growth suppression of glioma cells by PTEN requires a functional phosphatase catalytic domain, Proc Natl Acad Sci U S A. *94:* 12479-84, 1997.
36. Weidner, N., Semple, J. P., Welch, W. R., and Folkman, J. Tumor angiogenesis and metastasis--correlation in invasive breast carcinoma, N Engl J Med. *324:* 1-8, 1991.
37. Zundel, W., Schindler, C., Haas-Kogan, D., Koong, A., Kaper, F., Chen, E., Gottschalk, A. R., Ryan, H. E., Johnson, R. S., Jefferson, A. B., Stokoe, D., and Giaccia, A. J. Loss of PTEN facilitates HIF-1-mediated gene expression, Genes Dev. *14:* 391-396, 2000.

38. Zhong, H., Chiles, K., Feldser, D., Laughner, E., Hanrahan, C., Georgescu, M. M., Simons, J. W., and Semenza, G. L. Modulation of hypoxia-inducible factor 1alpha expression by the epidermal growth factor/phosphatidylinositol 3-kinase/PTEN/AKT/FRAP pathway in human prostate cancer cells: implications for tumor angiogenesis and therapeutics [In Process Citation], Cancer Res. *60:* 1541-5, 2000.
39. No, D., Yao, T. P., and Evans, R. M. Ecdysone-inducible gene expression in mammalian cells and transgenic mice, Proc Natl Acad Sci U S A. *93:* 3346-51, 1996.
40. Sheibani, N. and Frazier, W. A. Thrombospondin-1, PECAM-1, and regulation of angiogenesis, Histol Histopathol. *14:* 285-94, 1999.
41. Hsu, S. C., Volpert, O. V., Steck, P. A., Mikkelsen, T., Polverini, P. J., Rao, S., Chou, P., and Bouck, N. P. Inhibition of angiogenesis in human glioblastomas by chromosome 10 induction of thrombospondin-1, Cancer Res. *56:* 5684-91, 1996.
42. Tamura, M., Gu, J., Matsumoto, K., Aota, S., Parsons, R., and Yamada, K. M. Inhibition of cell migration, spreading, and focal adhesions by tumor suppressor PTEN, Science. *280:* 1614-7, 1998.
43. Holland, E. C., Celestino, J., Dai, C., Schaefer, L., Sawaya, R. E., and Fuller, G. N. Combined activation of ras and akt in neural progenitors induces glioblastoma formation in mice [In Process Citation], Nat Genet. *25:* 55-7, 2000.
44. Lawler, J., Sunday, M., Thibert, V., Duquette, M., George, E. L., Rayburn, H., and Hynes, R. O. Thrombospondin-1 is required for normal murine pulmonary homeostasis and its absence causes pneumonia, J Clin Invest. *101:* 982-92, 1998.
45. Dawson, D. W., Volpert, O. V., Pearce, S. F., Schneider, A. J., Silverstein, R. L., Henkin, J., and Bouck, N. P. Three distinct D-amino acid substitutions confer potent antiangiogenic activity on an inactive peptide derived from a thrombospondin-1 type 1 repeat, Mol Pharmacol. *55:* 332-8, 1999.
46. Crawford, S. E., Stellmach, V., Murphy-Ullrich, J. E., Ribeiro, S. M., Lawler, J., Hynes, R. O., Boivin, G. P., and Bouck, N. Thrombospondin-1 is a major activator of TGF-beta1 in vivo, Cell. *93:* 1159-70, 1998.
47. Dawson, D. W., Pearce, S. F., Zhong, R., Silverstein, R. L., Frazier, W. A., and Bouck, N. P. CD36 mediates the In vitro inhibitory effects of thrombospondin-1 on endothelial cells, J Cell Biol. *138:* 707-17, 1997.
48. Gao, A. G., Lindberg, F. P., Finn, M. B., Blystone, S. D., Brown, E. J., and Frazier, W. A. Integrin-associated protein is a receptor for the C-terminal domain of thrombospondin, J Biol Chem. *271:* 21-4, 1996.
49. Jimenez, B., Volpert, O. V., Crawford, S. E., Febbraio, M., Silverstein, R. L., and Bouck, N. Signals leading to apoptosis-dependent inhibition of neovascularization by thrombospondin-1, Nat Med. *6:* 41-8, 2000.
50. Good, D. J., Polverini, P. J., Rastinejad, F., Le Beau, M. M., Lemons, R. S., Frazier, W. A., and Bouck, N. P. A tumor suppressor-dependent inhibitor of angiogenesis is immunologically and functionally indistinguishable from a fragment of thrombospondin, Proc Natl Acad Sci U S A. *87:* 6624-8, 1990.
51. Brooks, P. C. Role of integrins in angiogenesis, Eur J Cancer. *32A:* 2423-9, 1996.
52. Tohma, Y., Gratas, C., Biernat, W., Peraud, A., Fukuda, M., Yonekawa, Y., Kleihues, P., and Ohgaki, H. PTEN (MMAC1) mutations are frequent in primary glioblastomas (de novo) but not in secondary glioblastomas, J Neuropathol Exp Neurol. *57:* 684-9, 1998.
53. Gu, J., Tamura, M., and Yamada, K. M. Tumor suppressor PTEN inhibits integrin- and growth factor-mediated mitogen-activated protein (MAP) kinase signaling pathways, J Cell Biol. *143:* 1375-83, 1998.
54. Adey, N. B., Huang, L., Ormonde, P. A., Baumgard, M. L., Pero, R., Byreddy, D. V., Tavtigian, S. V., and Bartel, P. L. Threonine phosphorylation of the MMAC1/PTEN PDZ binding domain both inhibits and stimulates PDZ binding, Cancer Res. *60:* 35-7, 2000.
55. Trueblood, C. E., Boyartchuk, V. L., Picologlou, E. A., Rozema, D., Poulter, C. D., and Rine, J. The CaaX proteases, afc1p and rce1p, have overlapping but distinct substrate specificities [In Process Citation], Mol Cell Biol. *20:* 4381-92, 2000.
56. Kohn, A. D., Barthel, A., Kovacina, K. S., Boge, A., Wallach, B., Summers, S. A., Birnbaum, M. J., Scott, P. H., Lawrence, J. C., Jr., and Roth, R. A. Construction and characterization of a conditionally active version of the serine/threonine kinase Akt, J Biol Chem. *273:* 11937-43, 1998.

57. Kohn, A. D., Summers, S. A., Birnbaum, M. J., and Roth, R. A. Expression of a constitutively active Akt Ser/Thr kinase in 3T3-L1 adipocytes stimulates glucose uptake and glucose transporter 4 translocation, J Biol Chem. *271:* 31372-8, 1996.
58. Zhou, X. P., Li, Y. J., Hoang-Xuan, K., Laurent-Puig, P., Mokhtari, K., Longy, M., Sanson, M., Delattre, J. Y., Thomas, G., and Hamelin, R. Mutational analysis of the PTEN gene in gliomas: molecular and pathological correlations, Int J Cancer. *84:* 150-4, 1999.
59. Georgescu, M. M., Kirsch, K. H., Akagi, T., Shishido, T., and Hanafusa, H. The tumor-suppressor activity of PTEN is regulated by its carboxyl- terminal region, Proc Natl Acad Sci U S A. *96:* 10182-7, 1999.
60. Di Cristofano, A. and Pandolfi, P. P. The multiple roles of PTEN in tumor suppression, Cell. *100:* 387-90, 2000.
61. Park, R. K., Erdreich-Epstein, A., Liu, M., Izadi, K. D., and Durden, D. L. High affinity IgG receptor activation of Src family kinases is required for modulation of the Shc-Grb2-Sos complex and the downstream activation of the nicotinamide adenine dinucleotide phosphate (reduced) oxidase, J Immunol. *163:* 6023-34, 1999.
62. Leslie, N. R., Gray, A., Pass, I., Orchiston, E. A., and Downes, C. P. Analysis of the cellular functions of PTEN using catalytic domain and C- terminal mutations: differential effects of C-terminal deletion on signalling pathways downstream of phosphoinositide 3-kinase, Biochem J. *346 Pt 3:* 827-33, 2000.
63. Mira, J. P., Benard, V., Groffen, J., Sanders, L. C., and Knaus, U. G. Endogenous, hyperactive Rac3 controls proliferation of breast cancer cells by a p21-activated kinase-dependent pathway, Proc Natl Acad Sci U S A. *97:* 185-9, 2000.
64. Durden, D. L., Rosen, H., Michel, B. R., and Cooper, J. A. Protein tyrosine phosphatase inhibitors block myeloid signal transduction through the Fc gamma RI receptor, Exp Cell Res. *211:* 150-62, 1994.
65. Dameron, K. M., Volpert, O. V., Tainsky, M. A., and Bouck, N. Control of angiogenesis in fibroblasts by p53 regulation of thrombospondin-1, Science. *265:* 1582-4, 1994.
66. Ozes, O. N., Mayo, L. D., Gustin, J. A., Pfeffer, S. R., Pfeffer, L. M., and Donner, D. B. NF-kappaB activation by tumour necrosis factor requires the Akt serine- threonine kinase [see comments], Nature. *401:* 82-5, 1999.
67. Biggs, W. H., 3rd, Meisenhelder, J., Hunter, T., Cavenee, W. K., and Arden, K. C. Protein kinase B/Akt-mediated phosphorylation promotes nuclear exclusion of the winged helix transcription factor FKHR1, Proc Natl Acad Sci U S A. *96:* 7421-6, 1999.
68. Medema, R. H., Kops, G. J., Bos, J. L., and Burgering, B. M. AFX-like Forkhead transcription factors mediate cell-cycle regulation by Ras and PKB through p27kip1, Nature. *404:* 782-7, 2000.
69. Giaccia, A. J. and Kastan, M. B. The complexity of p53 modulation: emerging patterns from divergent signals, Genes Dev. *12:* 2973-83, 1998.
70. Shaulian, E., Resnitzky, D., Shifman, O., Blandino, G., Amsterdam, A., Yayon, A., and Oren, M. Induction of Mdm2 and enhancement of cell survival by bFGF, Oncogene. *15:* 2717-25, 1997.
71. Besson, A., Robbins, S. M., and Yong, V. W. PTEN/MMAC1/TEP1 in signal transduction and tumorigenesis, Eur J Biochem. *263:* 605-11, 1999.
72. Gu, J., Tamura, M., Pankov, R., Danen, E. H., Takino, T., Matsumoto, K., and Yamada, K. M. Shc and FAK differentially regulate cell motility and directionality modulated by PTEN, J Cell Biol. *146:* 389-403, 1999.
73. Maier, D., Jones, G., Li, X., Schonthal, A. H., Gratzl, O., Van Meir, E. G., and Merlo, A. The PTEN lipid phosphatase domain is not required to inhibit invasion of glioma cells, Cancer Res. *59:* 5479-82, 1999.
74. Hu, L., Zaloudek, C., Mills, G. B., Gray, J., and Jaffe, R. B. In vivo and in vitro ovarian carcinoma growth inhibition by a phosphatidylinositol 3-kinase inhibitor (LY294002), Clin Cancer Res. *6:* 880-6, 2000.
75. Baumann, P. and West, S. C. DNA end-joining catalyzed by human cell-free extracts, Proc Natl Acad Sci U S A. *95:* 14066-70, 1998.
76. Vlahos, C. J., Matter, W. F., Hui, K. Y., and Brown, R. F. A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4- morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002), J Biol Chem. *269:* 5241-8, 1994.

77. Erdreich-Epstein, A., Shimada, H., Groshen, S., Liu, M., Metelitsa, L. S., Kim, K. S., Stins, M. F., Seeger, R. C., and Durden, D. L. Integrins alpha(v)beta3 and alpha(v)beta5 are expressed by endothelium of high-risk neuroblastoma and their inhibition is associated with increased endogenous ceramide, Cancer Res. *60:* 712-21, 2000.

INDIANA UNIVERSITY

June 26, 2000

Donald L Durden MD PhD
Associate Professor, Pediatrics and
Biochemistry
SCHOOL OF MEDICINE IU School of Medicine
Indianapolis, IN 46204

Dear Don:

I am pleased to collaborate with your laboratory to study the connection between PTEN/AKT and the p53/MDM2 pathways as it relates to the control of angiogenesis in brain tumors. As discussed we will provide expertise and reagents to transfer the PTEN gene into human glial tumor cell lines including retroviral vectors and other types of vectors. We look forward to a fruitful collaboration which is currently ongoing in this area of investigation.

I look forward to this exciting collaboration.

Sincerely,

David A Williams MD

David A. Williams, MD
Frieda and Albrucht Kipp Professor of Pediatrics
And Professor of Medical and Molecular Genetics
Indiana University School of Medicine
Associate Investigator, Howard Hughes Medical Institute
Indianapolis, IN

HERMAN B WELLS CENTER
FOR PEDIATRIC RESEARCH

James Whitcomb Riley
Hospital for Children
Indiana University
Medical Center
Cancer Research Institute
1044 W. Walnut Street
Room 402
Indianapolis, Indiana
46202-5225

317-274-8900
Fax 317-274-8679

Cold Spring Harbor Laboratory
P.O. Box 100, 1 Bungtown Road
Cold Spring Harbor, New York 11724

June 22, 2000

Donald L Durden MD PhD
Associate Professor, Pediatric and
Biochemistry and Molecular Biology
IU School of Medicine
Indianapolis, IN 46204

Dear Don:

I am pleased to continue to collaborate with your laboratory to study the connection between PTEN/AKT signaling pathways and glioma-induced angiogenesis. As discussed, I will continue to provide expertise and reagents to study PTEN in glial cells. We will provide your laboratory with additional mutants of PTEN and activated mutants of AKT and PI-3 kinase as well as PDK1 reagents. We look forward to continuing to work with you in a fruitful collaboration in this exciting area of investigation.

Yours sincerely,

Nicholas K. Tonks PhD
Professor
Tel: (516) 367-8846
Fax: (516) 367-6812
Email: tonks@cshl.org

INDIANA UNIVERSITY

June 22, 2000

Donald L Durden MD PhD
Associate Professor, Pediatric and
Biochemistry
SCHOOL OF MEDICINE IU School of Medicine

Dear Don:

I am pleased to collaborate with your laboratory to study the connection between PTEN/AKT signaling pathways and brain tumor progression. As discussed, we will provide expertise and reagents to study the expression of human glial tumors including an archival source of more than 2500 pediatric and adult tumor blocks for PTEN RT PCR. We have already worked out the conditions for immunohistochemical staining for Ki67, p53 and PTEN in combination with other routine stains which include H & E, GFAP, CD31. We will develop the immunohistochemical stains for MDM2 and EGF receptor expression in low and high-grade gliomas. We have agreement from our neurosurgical colleagues to obtain fresh flash frozen tumor tissue for immediate analysis as defined in the grant by Dr. Durden.

We look forward to this exciting collaboration which is currently ongoing between our laboratory and Dr Durdens group.7y

Sincerely,

Biagio Azzarelli

Biagio Azzarelli MD
Professor,
Department of Neuropathology
Indiana University
School of Medicine
Indianapolis, IN

DEPARTMENT OF PATHOLOGY
& LABORATORY MEDICINE

DIVISION OF NEUROPATHOLOGY

Medical Science Building A142
Indiana University
Medical Center
635 Barnhill Drive
Indianapolis, Indiana
46202-5120

317-274-8577
317-274-8578

INDIANA UNIVERSITY

June 26, 2000

Donald L Durden MD PhD
Associate Professor, Pediatric and
Biochemistry
SCHOOL OF MEDICINE IU School of Medicine

Dear Don:

I am pleased to collaborate with your laboratory to study the connection between PTEN/AKT signaling and the NFKB and p53/MDM2 signaling pathways. As discussed I will provide expertise and reagents to study p53 dependent transcription in order to examine the connection between the MDM2/p53 and PTEN/AKT pathways. We look forward to a fruitful collaboration which is currently ongoing in this exciting area of investigation. We are happy to support your work.

I look forward to this exciting collaboration.

Sincerely

David Donner

David B. Donner PhD
Professor, Microbiology and Immunology
Member, Walther Oncology Center
Indiana University
School of Medicine
Indianapolis, IN

WALTHER ONCOLOGY CENTER

R4 302
1044 W. Walnut Street
Indianapolis, Indiana
46202-5121

317-274-7501
317-274-7502
Fax: 317-274-7592

CHECKLIST

TYPE OF APPLICATION

☐ NEW application. *(This application is being submitted to the PHS for the first time).*

☐ REVISION of application number:_____
(This application replaces a prior unfunded version of a new, competing continuation, or supplemental application.)

☒ COMPETING CONTINUATION of grant number: R01 CA75637
(This application is to extend a funded grant beyond its current project period.)

INVENTIONS AND PATENTS (Competing continuation only)
☑ No ☐ Previously reported
☐ Yes, if yes ☐ Not previously reported

☐ SUPPLEMENT to grant number:_____
(This application is for additional funds to supplement a currently funded grant.)

☐ CHANGE of principal investigator/program director.
Name of former principal investigator/program director:_____

☐ FOREIGN application, city and country of birth and present citizenship of principal investigator/program director. *(This information is required by the U.S. Department of State.)*_____

1. ASSURANCES/CERTIFICATIONS

The following assurances/certifications are made by the signature of the Official Signing for Applicant Organization on the Face Page of the application. Descriptions of individual assurances/certifications begin on page 27 of Section III. If unable to certify compliance where applicable, provide an explanation and place it after this page.

*Human Subjects; *Vertebrate Animals; *Debarment and Suspension; *Drug-Free Workplace (applicable to new [Type 1] or revised [Type 1] applications only); *Lobbying; *Delinquent Federal Debt; *Research Misconduct; *Civil Rights (Form HHS 441 or HHS 690); *Handicapped Individuals (Form HHS 641 or HHS 690); *Sex Discrimination (Form HHS 639-A or HHS 690); *Age Discrimination (Form HHS 680 or HHS 690); *Financial Conflict of Interest

2. PROGRAM INCOME *(See instructions, page 20.)*
All application must indicate (Yes or No) whether program income is anticipated during the period(s) for which grant support is requested.

Budget Period	Anticipated Amount	Source(s)

3. INDIRECT COSTS

Indicate the applicant organization's most recent indirect cost rate established with the appropriated DHHS Regional Office, or, in the case of for profit organizations, the rate established with the appropriate PHS Agency Cost Advisory Office. If the applicant organization is in the process of initially developing or renegotiating a rate, or has established a rate with another Federal agency, it should , immediately upon notification that an award will be made, develop a tentative indirect cost rate proposal. That is to be based on its most recently completed fiscal year in accordance with the principles set forth in the pertinent *DHHS Guide for Establishing Indirect Cost Rates*, and submitted to the appropriate DHHS Regional Office or PHS Agency Cost Advisory Office. Indirect costs will ***not*** be paid on foreign grants, construction grants, grants to Federal organizations, and grants to individuals, and conference grants. Follow any additional instructions provided for Research Career Development Awards, Institutional National Research Service Awards, and the specialized grant applications.

☒ DHHS Agreement dated: 10/9/97

☐ No Indirect Costs Requested.

☐ DHHS Agreement being negotiated with _____Regional Office.

☐ No DHHS Agreement, but rate established with _____ Date_____

CALCULATION* (The entire grant application, including the Checklist, will be reproduced and provided to peer reviewers as CONFIDENTIAL information. Supplying the following information on indirect costs is OPTIONAL for profit organizations.)

a. Initial budget period: Amount of base $ 291,611 x Rate applied 49 % = Indirect costs (1) $ 142,889

b. Entire proposed project period:
Amount of base $ 1,482,559 x Rate applied 49 % = Indirect costs (2) $ 726,454

(1) Add to total direct costs from form page 4 and enter new total on FACE PAGE, Item 7b.
(2) Add to total direct costs from form page 5 and enter new total on FACE PAGE, Item 8b.

*Check appropriate box(es):

☐ Salary and wages base ☒ Modified total direct costs base ☐ Other base (Explain below)

☐ Off-site, other special rate, or more than one rate involved (Explain below)

Explanation *(Attach separate sheet, if necessary.)*:

4. SMOKE-FREE WORKPLACE

Does your organization currently provide a smoke-free workplace and/or promote the nonuse of tobacco products or have plans to do so?
X Yes No (The response to this question has no impact on the review of funding of this application.)

Detail of Facilities and Administration Costs Calculation

Year 1 Overall Direct Costs	$291,611
Less Equipment	$0
Less Subcontract with UPA over $25,000	$0
Year 1 Base Costs	$291,611
Year 1 F & A Costs @ 49%	$142,889
Year 1 Total Costs	$434,500

Entire Period Direct Costs	$1,548,202
Less Equipment	$0
Less Subcontract with UPA over $25,000	$65,643
Entire Period Base Costs	$1,482,559
	x .49
Entire Period F & A Costs @ 49%	$726,454
Entire Period Total Costs	$2,274,656

Principal Investigator/Program Director *(Last, first, middle)*: DURDEN, DONALD L.

Competing Continuation Applications

PERSONNEL REPORT

All Key Personnel for the Current Budget Period

Name	Degree(s)	SSN	Role on Project (e.g. PI, Res. Assoc.)	Date of Birth (MM/DD/YY)	Annual % Effort
Donald L. Durden	Ph.D.		P.I.		20%
Angela Hatten	M.S.		Res. Analyst		55%

Appendix

A. Figure 8.

B. Manuscripts.

1. Wen, S., Stolarov, J., Myers, M.P., Su, J.D., Wigler, M.H., Tonks, N.K., **Durden, D.L.** PTEN controls the growth and angiogenic response of malignant gliomas. ***Nat. Genet.*** Submitted, 2000.

2. Erdreich-Epstein, A., Shimada, H., Groshen, S., Liu, M., Metelitsa, L.S., Kim, K.S., Stins, M.F., Seeger, R.C., **Durden, D.L.**. Integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ are expressed by endothelium of high-risk neuroblastoma and their inhibition is associated with increased endogenous ceramide. ***Cancer Res.***, 60:712-721, 2000.

3. Park, R.K., Erdreich-Epstein, A., Liu, M., Izadi, K.D., **Durden, D.L.** High affinity IgG receptor activation of Src family kinases is required for modulation of the Shc-Grb2-Sos complex and the downstream activation of the nicotinamide adenine dinucleotide phosphate (reduced) oxidase. 1999. ***J. Immun.***, 163:6023-6034, 1999.

4. Park, R.K., Izadi, K.D., Deo, Y.M., **Durden, D.L.** Role of Src in the modulation of multiple adaptor proteins in FcαRI oxidant signaling. ***Blood***, 94:2112-2120, 1999.

5. Erdreich-Epstein, A., Liu, M., Kant, A.M., Izadi, K.D., Nolta, J.A., **Durden, D.L.** Cbl functions downstream of Src kinases in FcγRI signaling in primary human macrophages. ***J. Leukocyte Biol.***, 65:523-534, 1999.

6. Izadi, K.D., Erdreich-Epstein, A., Liu, Y., **Durden, D.L.** Characterization of Cbl-Nck and Nck-Pak1 interactions in myeloid FcγRII signaling. ***Exp. Cell Res.***, 245:330-342, 1999.

7. Kyono, W.T., de Jong, R., Park, R.K., Liu, Y., Heisterkamp, N., Groffen, J., **Durden, D.L.** Differential interaction of Crkl with Cbl or C3G, Hef-1, and γ subunit immunoreceptor tyrosine-based activation motif in signaling of myeloid high affinity Fc receptor for IgG (FcγRI). ***J. Immun.***, 161:5555-5563, 1998.

8. Chu, J., Liu, Y., Koretzky, G.A., **Durden, D.L.** Slp-76-Cbl-Grb2-Shc interactions in FcγRI signaling. ***Blood***, 92:1697-1706, 1998.

9. Park, R.K., Kyoni, W.T., Liu, Y., **Durden, D.L.** CBL-GRB2 interaction in myeloid immunoreceptor tyrosine activation motif signaling. ***J. Immun.***, 160:5018-5027, 1998.

10. Erdreich-Epstein, A., Liu, M., Liu, Y., **Durden, D.L.** Protein tyrosine phosphatase inhibitors in FcγRI-induced myeloid oxidant signaling. ***Exp. Cell Res.***, 237:288-295, 1997.

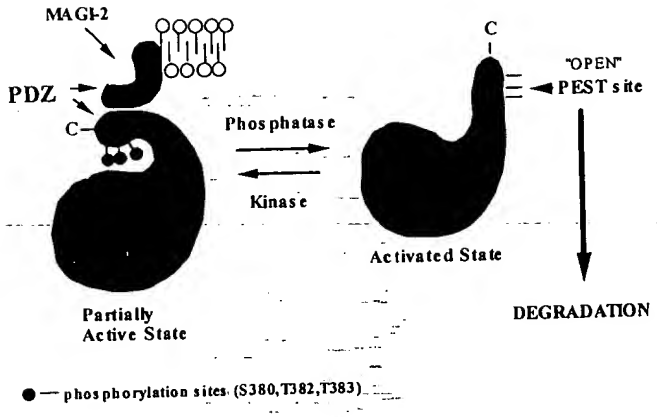

Figure 8. The C terminus of PTEN regulates protein stability and membrane association. This schematic shows the position of PTEN PDZ domain relative to 3 phosphorylation sites recently implicated in the regulation of PTEN. PTEN in the phosphorylated form is associated with MAGI-2 via one of its PDZ domains. The dephosphorylation of PTEN results in increased catalytic activity and "opens" a putative PEST site which may contribute to the degradation of the activated form of PTEN. We show the phosphorylation sites planned to be mutated in Aim 1 and our view of how PTEN may undergo autoregulation cycle within the cell.